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“Tumor-educated Mast Cells Induce Epithelial-to-Mesenchymal Transition and Expansion of Stem Cell Population via IL-8/CXCR1/CXCR2 Axis in Thyroid Cancer”

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“Tumor-educated Mast Cells Induce Epithelial-to-Mesenchymal Transition and Expansion of Stem Cell Population via IL-8/CXCR1/CXCR2 Axis in Thyroid Cancer”

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LIST OF ABBREVIATIONS

ABCG2: ATP-Binding Cassette, sub-family G, member 2

AKAP9: A Kinase Anchor Protein 9

AKT: acutely transforming retrovirus thymoma protein kinase

ALDH: Aldehyde Dehydrogenase

APS: Ammonium Persulfate

ATC: Anaplastic Thyroid Carcinoma

ATP: Adenosine Tri-Phosphate

BAD: Bcl-2-Associated Death promoter

BCR-ABL: Breakpoint Cluster Region-Abelson

BRAF: B-Rapidly Accelerated Fibrosarcoma

BSA: Bovine Serum Albumin

CAF: Cancer Asscіated Fibroblast

CCDC6: Coiled-Coil Domain Containing 6.

CSC: Cancer Stem Cell

DEPC: diethylpyrocarbonate

DMEM: Dulbecco's Modified Eagle Medium

DNA: Deoxyribonucleic Acid

dNTP: Deoxyribonucleotide triphosphate

EC: Extracellular domain

ECL: Enhanced ChemiLuminescence

ECM: Extracellular Membrane

EGF: Epidermal Growth Factor

ELISA: Enzyme-Linked Immunosorbent Assay

EMT: Epithelial-to Mesenchymal Transition

ERK: Extracellular-Signal-Regulated Kinase

FA: Follicular Adenoma

FBS: Fetal Bovine Serum

FGF: Fibroblast Growth Factor

FMTC: Familiar Medullary Thyroid Carcinoma

FTC: Follicular Thyroid Carcinoma

GAPDH: Glyceraldehyde 3 phosphate dehydrogenase

GDNF: Glial-Derived Neurotrophic Factor

GDP: Guanosine DiPhosphate

GFP: Green Fluorescent Protein

GFR α: GDNF Family Receptor α

GPI: GlycosylPhosphatidylInositol

GSK-3β: Glycogen Synthase Kinase 3β

GTP: Guanosine TriPhosphate

HCV: Hepatitis C Virus

HEPES: N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid

HGF: Hepatocyte Growth Factor

HMC-1: Human Mast Cell line 1

HT: Hashimoto's Thyroiditis

IF: Immunofluorescence

IgE: Immunoglobulin E

IGF: Insulin-like Growth Factor

IL-1β: Interleukin-1β

IL-6: Interleukin-6

IL-8: Interleukin-8

JAM: Junctional Adhesion Molecule

LSM: Laser Scanning Microscope

LTC4: Leukotriene C4

LTD4: Leukotriene D4

LTE4: Leukotriene E4

MC: Mast Cell

MC CM: Mast Cell Conditioned Medium

MEK: MAP and ERK Kinase

MEN 2: Multiple Endocrine Neoplasia type 2

MET: Mesenchymal- Epithelial Transition

$MgCl_2$: Magnesium Chloride

MMPs: Metalloproteinases

mRNA: messenger RNA

MTC: Medullary Thyroid Carcinoma

MT-MMPs: Membrane Type- Metalloproteinases

mTOR: mammalian Target Of Rapamycin

MUPP: Multi-PDZ Domain Protein

Ncoa4: Nuclear receptor coactivator 4

NGF: Nerve Growth Factor

OCT-4: octamer-binding transcription factor-4

OPN: Osteopontin

PALS: Protein Associated with Lin Seven 1

PAR: Partitioning defective

PATj: Pals 1-associated tight junction protein

PAX8/PPARγ: Paired Box -8/Peroxisome Proliferator-Activated Receptor γ

PBS: Phosphate Buffered Saline

PDTC: Poorly Differentiated Thyroid Carcinoma

PDZ domain: Post synaptic density protein (PSD95), Drosophila discs large tumor suppressor (Dlg1), Zonula occludens-1 (ZO-1)

PGD2: Prostaglandin D2

PGE2: Prostaglandin E2

PH domain: Pleckstrin-Homology domain

PI3K: Phosphatidylinositol-3-Kinase

$PIP_{3:}$ phosphatidylinositol (3,4,5)-triphosphate

PKB: Protein Kinase B

PRO: Pro-peptide Region

PTC: Papillary Thyroid Carcinoma

Q-PCR: Quantitative Real Time Polymerase Chain Reaction

RAF: Rapidly Accelerated Fibrosarcoma

RAS: Rat Sarcoma

RET: Rearranged during Transfection

RFG: Ret Fused Gene

RH: Random Hexamers

RI: RNase Inhibitor

RNA: ribonucleic acid

ROS: Reactive Oxygen Species

RT: Room Temperature

RTK: Tyrosine Kinase Receptor

SCF: Stem Cell Factor

SDS: Sodium Dodecyl Sulfate

SDS-PAGE: Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis

SH3: Src Homology 3 domain

SIP1: Smad-Inter- acting Protein 1

SP: Signal Peptide

SRC: Sarcoma

STAT3: Signal Transducer and Activator of Transcription 3

T3: triiodothyronine

T4: thyroxine

TAM: Tumor Associated Macrophage

TC: Thyroid carcinoma

TCF/LEF: T Cell-Factor/Lymphoid-Enhancer-Factor

TEMED: Tetramethylethylenediamine

TGF- β: Transforming Growth Factor- β

TIMPs: Tissue Inhibitors of Metalloproteinases

Tjs: Tight junctions

TK: Tyrosine Kinase

TLRs: Toll Like Receptors

TM: Transmembrane domain

TNF- α: Tumor Necrosis Factor- α

TPR: Translocated Promoter Region

TrisHCl: tris(hydroxymethyl)-aminomethane-HCl

TRKA: Tyrosine Receptor Kinase A

TSH: Thyroid Stimulating Hormone

UTC: Undifferentiated Thyroid Carcinoma

VEGF: Vascular Endothelial Growth Factor

WDTC: Well-Differentiated Thyroid Carcinomas

ZEB: zinc finger E box-binding homeobox

ZO: Zonula Occludens

LIST OF PUBLICATIONS

1: Liotti F, **Visciano C**, Melillo RM. Inflammation in thyroid oncogenesis. Am J Cancer Res. 2:286-97. 2012. ISSN:2156-6976

2: **Visciano C**, Liotti F, Prevete N, de Paulis A, Marone G, Santoro M, Melillo RM. Tumor-educated Mast cells Induce Epithelial-to-Mesenchymal Transition and Expansion of Stem Cell Population via IL-8/CXCR1/CXCR2 Axis in Thyroid Cancer. *In preparation*

ABSTRACT

We previously demonstrated that human papillary thyroid carcinomas (PTCs) display a Mast Cell (MC) infiltrate correlated with extrathyroidal extension.
MCs (MC) are mainly found at the invasive front of human PTCs, where they may facilitate tumor cell migration and extracellular matrix degradation. The increased motility and invasiveness of tumor cells are reminiscent of the epithelial to mesenchymal transition (EMT). However, the MC-derived factors that mediate this activity and the mechanisms with which they enhance thyroid carcinoma (TC) invasive ability remain unidentified. Here, we report that MC-derived conditioned media (MC CM) induce the epithelial-to-mesenchimal transition (EMT) and the gain of stemness features of TC cells. MC CM-treated thyroid cancer (TC) cells down-regulate E-cadherin expression, an epithelial protein, while up-regulating EMT transcription factors (SNAIL, SLUG, ZEB1) and mesenchimal markers (vimentin). Here we identify interleukin 8 (CXCL8/IL-8) as the main mediator produced by MCs capable of inducing both EMT and stemness in TC cells. TC cells infact express both the IL-8 receptors, CXCR1 and CXCR2. Immune depletion of IL-8 from MC CM completely abolishes EMT and stemness. Treatment of TC cells with recombinant IL-8 induces these features, and blocking molecules targeting the IL-8/CXCR1/CXCR2 axis revert them. 850-5C cells, derived from a human anaplastic thyroid carcinoma (ATC), enforced to express IL-8, undergo EMT, display increased stemness, and are more efficient in tumor formation when xenografted in immunodeficient mice with respect to parental cells. The transcription factor SLUG may be a critical mediator of IL-8-mediated biological effects in TC cells; in fact, IL-8 treatment causes persistent SLUG upregulation, and IL-8 expressing 850-5C cells constitutively express high levels of SLUG. We generated TPC-1 cells, derived from a human PTC, with enforced expression of SLUG. These cells underwent EMT and exhibited stemness features, including the capability to form spheres in low-adherence condition. By analysing a panel (n=30) of human TC surgical samples, we observed that the expression of the stemness marker OCT4 is significantly associated with tumor size and with MC density. Furthermore, we observed that IL-8 expression correlates with the presence of lymph-node metastases and with SLUG expression.
The failure in the eradication of CSCs is likely one of the factors that limits the efficacy of current therapeutic approaches against cancer. Thus, novel CSCs-targeted treatments are needed. In this regard, our study provides the evidence that targeting MC-derived IL-8 may be employed to directly target CSCs in advanced thyroid cancer.

1. BACKGROUND

1.1 Thyroid Cancer

The thyroid gland develops in the embryo as a tubular evagination of the pharyngeal endoderm at the basis of the tongue. It is located in the neck, beside the trachea. The thyroid gland is composed by two distinct hormone-producing cell types: follicular cells and parafollicular *C* cells. Follicular cells comprise most of the epithelium and are responsible for iodine uptake and thyroid hormone synthesis. *C* cells are scattered intrafollicular or parafollicular cells and are dedicated to the production of the calcium-regulating hormone calcitonin. Extimates of 2012 reveal thyroid cancer (TC) as the fifth expected malignancy in American women and is the most common malignancy of the endocrine system (Peter M Sadow and William C. Faquin 2012). More than 95% of TC are derived from follicular cells while a minority of tumors (3%), referred to as medullary thyroid carcinoma, are of *C*-cell origin.
TC affects women more often than men and usually occurs in people between the ages of 25 and 65 years. The incidence of this malignancy has been increasing over the last decade (Pellegritti G et al. 2013).
Patients with a history of radiation administered in infancy and childhood for benign conditions of the head and neck, such as enlarged thymus, acne, or tonsillar or adenoidal enlargement, have an increased risk of cancer as well as other abnormalities of the thyroid gland (Ciampi et al. 2005). Other risk factors for the development of TC include a history of goiter, family history of thyroid disease, female gender.
Thyroid carcinomas are broadly divided into well-differentiated, poorly differentiated and undifferentiated types on the basis of histological and clinical parameters. Differentiated tumors (papillary or follicular) are highly treatable and usually curable. Poorly differentiated tumors (medullary and anaplastic) are much less common, are aggressive, metastasize early, and have a much poorer prognosis (DeLellis et al. 2004). Papillary thyroid carcinoma (PTC) is the most common thyroid malignancy; it accounts for 75 to 80% of all thyroid cancers, showing an incidence peak at 40-50 years. A well established cause of PTC is the exposure to ionizing radiations, especially in the neck region; in fact, about 2-4% of the patients irradiated to treat deseases such as acne or enlarged thymus, develop a differentiated thyroid carcinoma after about 20-30 years. Accordingly, the frequency of PTC is dramatically increased in the children exposed to the massive release of radionuclides that followed the explosion of the nuclear reactor in Chernobyl in 1986 (DeLellis, 2006). PTC shows typically multicentricity and a tendency to spread into lymphatic vessels; regional node metastases at presentation are found in a significant

proportion of cases. There are several PTC variants including solid-follicular, follicular, tall-cell, hurthle cell variants (Ostrowski et al. 1996). Follicular thyroid carcinoma (FTC) is less frequent than PTC and represents about 10-30% of thyroid cancers; it is particularly linked to dietary iodine deficiency (Williams et al.1997) and both iodine deficiency and genetic influences could account for its link with a history of nodular goiter (Ron E et al. 1987). FTC shows variable morphology ranging from well-formed colloid-containing follicles, to solid or trabecular growth pattern. Therapy for both PTC and FTC consists in surgery followed by metabolic treatment with I^{131}. Prognosis is very good with a survival rate at 10 years ranging from 90 to 98%.

Anaplastic thyroid carcinoma (ATC) is the most aggressive type of thyroid cancer. ATC cells are extremely abnormal and spread rapidly to other parts of the body. ATC make up only about 1% of all thyroid cancers and typically spreads beyond the thyroid by direct local extension. Metastasis to regional nodes are also common but their presence is often masked by the presence of extensive soft tissue invasion. No effective therapy is known for ATC and prognosis is very unfavorable with a mean survival time of less than one year (Giuffrida et al. 2000). Poorly differentiated thyroid carcinoma (PDTC) is a recent diagnosis for rare aggressive thyroid tumors that represent the bridge between more well differentiated malignancies of thyrocytes and the undifferentiated thyroid carcinomas (Siironen P et al. 2010).

PDTCs demonstrate solid, trabecular, and insular components, the absence of conventional nuclear patterns of papillary carcinoma, and the presence of at least one of the following features: convoluted nuclei, mitotic activity, or necrosis (Katsuhiro Tanaka et al. 2011). Many reports have demonstrated a poorer survival among PDTC patients than among those with well-differentiated carcinoma (WDTC)(M. Decaussin et al. 2002, M. Volante et al. 2004, T. Nishida et al. 1999).

Despite favorable prognosis of WDTC, ~5% of them progress to radioactive iodine-refractory (RAIR), which commonly leads to death within 5 years (Pacini F et al. 2012). I^{131} RAIR can be defined as an inability of malignant/metastatic tissue to take up I^{131} from the start of treatment or loss of ability to take up I^{131} after previous evidence of uptake. It is detected by a combination of imaging modalities, including an I^{131} whole body scan showing at least one lesion that does not take up I^{131}, or clinical evidence that I^{131} is no longer providing benefit to the patient (David G. Pfister and James A. Fagin 2008). Standard cytotoxic chemoterapy has limited efficacy, making introduction in clinical trials of novel targeted therapies (Pacini F et al. 2012),

Medullary thyroid carcinoma (MTC) develops from neuroendocrine C cells of the thyroid, producing calcitonin (CT). About 5 to 7% of all TC are MTC that can be sporadic or familial as part of the Multiple Endocrine Neoplasia type 2 syndromes. MEN2 syndromes are inherited cancer disorders including three types: MEN2A, characterized by MTC, pheochromocytoma and parathyroid adenoma; MEN2B characterized by MTC, pheochromocytoma and additional tumors such as neuromas and ganglioneuromas of the gut; Familial Medullary

Thyroid Carcinoma or FMTC whose only feature is MTC. MEN2 is inherited as a highly penetrant mendelian tract and this genetic transmission is due to gain-of-function mutations of the RET gene. MTC tends to metastasize to lymph-nodes and distant organs, and its treatment consists in surgical removal of the lesion. MTC are fairly resistant to most chemotherapeutic agents (Core et al.2003). *Fig.1*

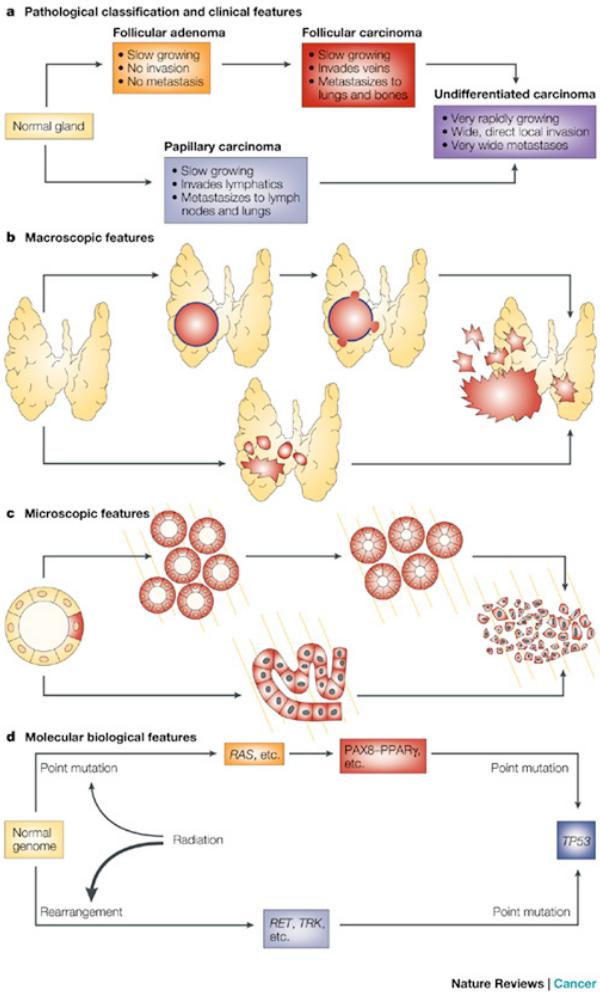

Figure 1: Features of thyroid tumors (Modified from Williams 2002).

1.2 Molecular genetics of thyroid tumors

PTC

Several studies on thyroid tumors have allowed the identification of many genetic alterations. In particular, four genetic lesions, at the somatic level, are

associated with PTC. They include chromosomal aberrations targeting the RET or TRKA tyrosine kinase receptors and point mutations in RAS or BRAF genes. *RET(Rearranged during Transfection)* and *NTRK1* are involved in the pathogenesis of papillary thyroid carcinoma (PTC), with *RET* alterations being more common than *NTRK1* rearrangements (Santoro M et al. 1995, Bongarzone I et al. 1999). Both genes code for neurotrophic growth factor receptors with tyrosine kinase activity. Approximately 20–40% of PTCs exhibit rearrangements of one of these genes with other coding sequences, forming chimeric oncogenes, including *RET/PTC.* In RET/PTC rearrangements, fusion with protein partners, possessing protein-protein interaction domains, provides RET/PTC proteins the ability to constitutively dimerize, thereby resulting in ligand-independent activation of c-Ret tyrosine kinase activity (Santoro et al. 1995). RET/PTC1, the CCDC6-RET fusion, and RET/PTC3, the NCOA4-RET fusion, are the most prevalent variants. RET/PTC3 is frequently found in radiation-associated-PTC occurred after the Chernobyl accident.

The RAS family of proto-oncogenes includes *KRAS,* and *HRAS and NRAS,* all of which are membrane-bound GTP/GDP-binding (G) molecules. These proteins transfer signals from activated tyrosine receptors to pathways that lead to the synthesis of key proteins involved in cell growth, proliferation and survival. Although *Ras* mutations have been observed in 10–15% of PTCs, they are more commonly associated with FTC and the follicular variant of papillary cancer (Zhu Z et al. 2003). ARaf, BRaf and CRaf are isoforms of the mammalian Raf serine–threonine kinase that participate in the RAF/MEK/MAPK pathway. *BRAF* mutations, observed in nearly 40% of PTCs , have been associated with a more aggressive course of disease (Elisei R et al. 2008). A Glutamine for Valine substitution at residue 600 (V600E) in the activation segment of the kinase accounts for more than 90% mutations of BRAF in PTC (Kimura et al. 2003; Cohen et al. 2003; Soares et al. 2003). This mutation enhances BRAF activity through disruption of the autoinhibited state of the kinase. In human PTC, the genetic alterations of RET/PTC, RAS and BRAF are mutually exclusive, suggesting the existence of a common signaling cascade; moreover, mutations at more than one of these sites are unlikely to provide an additional biological advantage (Kimura et al. 2003, Cohen et al. 2003, Soares et al. 2003).

Among the other genetic anomalies identified in patients with PTC are single-nucleotide polymorphisms in genes that regulate transcription, upregulation of microRNAs that modify gene expression and increased methylation of tumor suppressor genes (Hu S et al. 2006). In addition, familial PTC has been associated with abnormalities in the short arm of chromosome 19 and the long arms of chromosomes 1, 2 and 8 (Canzian F et al. 1998, McKay JD et al. 2001), as well as with short telomeres in the germline (Capezzone M et al. 2011).

FTC

Follicular thyroid carcinoma (FTC) is also a WDTC developing from thyroid cells. In this carcinoma the presence of K-, H-, and NRAS mutations is quite common. More recently, it has been shown that a quite high proportion of FTC carries a translocation involving the *PAX8* promotor gene on the long arm of chromosome 2 and the *PPAR*-γ gene on the short arm of chromosome 3 [t(2;3) (q13;p25)], resulting in the fusion of their DNA-binding domains. PAX8 is a critical regulator of the development and differentiation of follicular epithelium, whereas PPAR-γ is a nuclear hormone receptor, normally not expressed at high concentrations in thyroid, which is involved in carbohydrate and lipid metabolism and inflammation (Kroll et al. 2000).

PDTC and ATC

PDTC is characterized by mutations in *BRAF*, *N*, *K*, and *HRAS*, as well as translocation of RET/ PTC1, RET/PTC3.

Point mutations in the *RAS* oncogene and in the tumor suppressor p53 have been described in ATC (Garcia-Rostan et al. 2005, Donghi et al. 1993, Fagin 1993). It is well known that p53 safeguards the cell cycle, the DNA repair and the apoptotic processes; Garcia-Rostan and colleagues found somatic mutations within the PI3K catalytic subunit in 23% of analyzed ATC as well as activating point mutations of β-catenin (Garcia-Rostan et al. 1999). Mutations of BRAF gene have been detected in anaplastic carcinomas, suggesting that some ATC may arise from a preexisting WDTC, while others may arise *de novo* (Nikiforova MN et al. 2003).

MTC

MTC can be sporadic or it could be one of the lesions that characterize the autosomal dominant MEN 2 syndromes (MEN2A, MEN2B and FMTC). MEN 2 syndromes are caused by germline point mutations that convert *RET* into a dominant oncogene. MEN2 patients are invariably affected by Medullary Thyroid Carcinoma (MTC), a malignant tumour arising from calcitonin-secerning C cells of the thyroid. Additional features can be present in MEN2A (pheochromocytoma and parathyroid adenoma) and MEN2B (pheochromocytoma, mucosal neuroma and ganglioneuroma of the intestine) (Brandi et al. 2001). In more than 80% of cases, MEN2B is caused by the Met918Thr substitution in the kinase domain of the receptor. In MEN2A and most FMTC patients, mutations affect one cysteine of the extracellular cysteine-rich domain of RET (609, 611, 618, 620, 630, 634) that can change to different residues. A significant genotype-phenotype correlation is observed. In particular, about 90% of MEN2A patients have Cys634 mutation, and this mutation is highly predictive of the presence of pheochromocytoma and parathyroid hyperplasia. (Santoro et al. 1995; Carlomagno et al. 1997). RET activation by mutations targeting the intracellular domain is less understood

(Santoro et al. 1995, Iwashita et al. 1999) but it can be envisaged that a modification of the structure of the kinase may switch on its enzymatic function. Mutations in *HRAS* and *KRAS* genes were recently found in a significant proportion of non-*RET*-mutated MTC, suggesting that *RAS* mutations could represent alternative genetic events in sporadic MTC tumorigenesis. This observation could be of value for defining therapeutic strategies in non-*RET*-mutated tumors (A. Boichard et al. 2012).

1.3 Inflammation and thyroid cancer

The relationship between cancer and inflammation has been observed first in 1863 when Rudolf Virchow, following the observation of leukocytes in neoplastic tissues, hypothesized that chronic inflammation could contribute to the tumorigenic process. In the following decades, several lines of evidence suggested a strong association between chronic inflammation and increased susceptibility to neoplastic transformation and development. It has been estimated that up to 20% of all tumors arise from conditions of persistent inflammation, such as chronic infections or autoimmune diseases. Indeed, the association between cervical cancer and papilloma virus, gastric cancer and *Helicobacter pylori* induced gastritis, esophageal adeno-carcinoma and Barrett's metaplasia, hepatocellular carcinoma, hepatitis B and C viral infections, and many others are well recognized (Anand P. et al. 2008). The mediators and cellular effectors of inflammation are important constituents of the local environment of tumors. In some types of cancer, inflammatory conditions are present before a malignant change occurs. Conversely, in other types of cancer, an oncogenic change induces an inflammatory microenvironment that promotes the development of tumours. Regardless of its origin, chronic inflammation in the tumour microenvironment has many tumour-promoting effects such as proliferation and survival of malignant cells, promotes angiogenesis and metastasis, subverts adaptive immune responses, and alters responses to hormones and chemotherapeutic agents. The inflammatory microenvironment of neoplastic tissues is characterized by the presence of host leukocytes both in the supporting stroma and among the tumor cells, with macrophages, dendritic cells, MCs, and T cells being differentially distributed (Balkwill and Mantovani, 2001). Several cytokines (TNF, IL-1, IL-6) and chemokines that are produced by the tumor cells and by leukocytes and platelets associated with the tumor have been found to be able to maintain the invasive phenotype (Coussens and Werb, 2002). Tumor-associated macrophages (TAMs) are a major component of the leukocyte infiltrate, initially recruited by inflammatory chemokines (e.g., CCL2) and then sustained by cytokines present in the tumor microenvironment (e.g., CSFs, VEGF-A). By producing cytokines such as TGF-β, IL-10, and M-CSF, TAMs promote tumor proliferation and progression and stroma deposition and, indeed, the density of TAMs is increased in advanced thyroid cancers (Ryder et al., 2008). The connection between inflammation and cancer can be viewed as consisting of

two pathways: an extrinsic pathway, driven by inflammatory conditions that increase cancer risk (such as inflammatory bowel disease); and an intrinsic pathway, driven by genetic alterations that cause inflammation and neoplasia (such as oncogenes). The intrinsic pathway was uncovered when addressing why inflammatory cells and mediators are present in the microenvironment of most, if not all, tumors and therefore are present in cases for which there is no epidemiological basis for inflammation. This finding raised the question of whether the genetic events that cause neoplasia in these cases are responsible for generating an inflammatory environment. This question has been addressed only recently, by using preclinical and clinical settings in which various oncogenetic mechanisms can be assessed. The intrinsic pathway is activated by genetic events that cause neoplasia. These events include the activation of various types of oncogene by mutation, chromosomal rearrangement or amplification, and the inactivation of tumor-suppressor genes. Cells that are transformed in this manner produce inflammatory mediators, thereby generating an inflammatory microenvironment in tumors for which there is no underlying inflammatory condition (for example, breast tumors). By contrast, in the extrinsic pathway, inflammatory or infectious conditions augment the risk of developing cancer at certain anatomical sites (for example, the colon, prostate and pancreas) (Colotta et al. 2009). Thyroid cancer obeys to these rules. First, an inflammatory component, which includes different leukocyte types, is frequently observed in thyroid tumors and the papillary histotype of thyroid cancer (PTC) is often associated with organ-specific autoimmune diseases, such as Hashimoto's thyroiditis and Grave's disease. Second, RET/PTC, RAS (V12) or BRAF (V600E), the most common oncogenes found in human PTC, trigger an inflammatory transcriptional program in thyreocytes (Melillo RM et al. 2005).

An inflammatory component, which includes different leukocyte types, is frequently observed in thyroid tumors as well: macrophages and immature dendritic cells accumulate in PTCs, both in tumoral stroma and at the invasive front; the prevalence of lymphocytic infiltrate is generally significantly higher in patients with PTC than in those with benign thyroid lesions, indicating that the presence of these cells might favor cancer development; PDCs and ATCs display an increased density of tumor-associated macrophages (TAM) with respect to PTC and FTC; moreover, TAM infiltration positively correlates with malignant progression of thyroid cancer. MCs (MC) also play an important role in thyroid neoplastic diseases, because PTCs, but not normal thyroid tissue, display an intense MC infiltrate whose intensity positively correlates with the invasive phenotype of TC (Liotti et al. 2012).

The intrinsic pathway of cancer-related inflammation is driven by the most frequent genetic alterations associated with thyroid cancer, such as RET and TRK rearrangements and BRAF and RAS mutations. It has been found that RET/PTC3 oncogene induces a proinflammatory transcriptional program, that includes several cytokines such as osteopontin (OPN), VEGF-A, CCL2, CXCL1 and CXCL10. Accordingly, Iwahashi showed that activation of RET

induces the production of high levels of IL-8, a pro-inflammatory, mitogenic and proangiogenic chemokine that contributes to several human cancer (Iwahashi et al. 2002). The proinflammatory properties of RET/PTC in thyroid might have a dual effect: on one side, molecules such as OPN, CXCL1, CXCL10, CCL2, GM-CSF can influence immune response to the tumor by recruiting and functionally regulating immune cells. On the other side, secreted cytokines and chemokines, such as OPN, CXCL1, CXCL10, and IL-1β can act as autocrine growth and survival factors for thyroid tumor cells, which express the cognate receptors on their plasma membrane (Liotti et al. 2012).

1.4 MC and thyroid cancer

MCs (MC) originate in the bone marrow and enter the circulation in immature form. Once settled into a tissue site, they mature, taking on characteristics specific for that tissue. There are two general types of MC: those found in connective tissue and those found in mucosal tissue. They have a relevant role in the pathogenesis of allergic diseases, being stimulated by the IgE-induced FcεRI receptor activation (Marone et al., 2005). Together with dendritic cells, MCs are among the first immune cell populations to interact with environmental antigens, allergens, bacteria, viruses and parasites (Galli et al., 2005). The c-kit receptor ligand, Stem Cell Factor (SCF), is the most relevant factor for human MC maturation and differentiation (Galli et al., 2005). MC immunological activation results in the secretion of several proinflammatory factors. Among the proteins secreted by activated MCs there are preformed factors (histamine, tryptase, chimase, carbossipeptidase A and proteoglycanes) stored in secretory granules and extracellularly released within few minutes, lipidic mediators (cyclooxygenases-PGD2 and PGE2- and the lipo-oxygenase -LTC4, LTD4, LTE4- metabolites), several cytokines (TNF- α, IL-13, IL-3, GM-CSF and IL-5) and chemokines (IL-8, CCL3/MIP-1α, CXCL1/GRO-α and CXCL10/IP-10). Relevantly, MCs secretory responses can be influenced by both genetic and environmental factors (Galli et al., 2005). MCs, as macrophages, are long surviving cells; their number increases in case of immediate hypersensitive reactions (rhinitis, eczema, asthma), of infections caused by intestinal worms, of chronic inflammation and/or tissue remodeling (rheumatoid arthritis, for example). It is well known that various types of hematologic malignancies and solid cancers are associated with increased MCs. In many tumors, MC counts have been reported to even correlate with tumor stage, prognosis and invasiveness. (Ribatti et al. 2011, Pittoni P et al. 2011, Melillo RM et al. 2010, Amini RM et al. 2007, Theoharides et al. 2004). A correlation between numbers of MCs and poor prognosis was demonstrated in human lymphoid neoplasms such as Hodgkin´s lymphoma (Molin D et al. 2002), B-cell non-Hodgkin's lymphoma (Ribatti D et al. 2000), primary cutaneous lymphoma (Rabenhorst A et al. 2012) and multiple myeloma (Nico B et al. 2008). Similar data were obtained in human solid cancers, such as

pancreatic cancer (Strouch MJ et al. 2010), hepatocarcinoma, cholangiocarcinoma (Tereda T et al. 2000), prostate cancer (Pittoni P et al. 2011) and melanoma (Toth-Jakatics R et al. 2000 and Ribatti D et al. 2003). In a recent study on human and murine prostate tumors, MCs were shown to increase upon disease. Experiments using MC-deficient mice demonstrated that MCs were necessary for development of transplanted well differentiated prostate tumors progression (Pittoni P et al. 2011). Similar results were obtained in pancreatic cancer confirming the essential role of MCs for invasion of tumors (Strouch MJ et al. 2010). Patients with pancreatic cancer showed elevated serum tryptase levels suggesting that increased numbers of MCs and MC-conditioned medium induced migration, proliferation and invasion of pancreatic cancer cell lines. On the other hand, pancreatic cancer cells stimulated migration of MCs (Strouch MJ et al. 2010). In murine pancreatic cancer, MC numbers were increased and correlated again with tumor grade and reduced survival (Strouch MJ et al. 2010). Supernatants of MCs were shown to induce cytokine release and proliferation of primary cutaneous lymphoma cells in vitro. Several recent studies also suggested indirect effects of MCs on tumors through interaction with other immune cells and blood vessels. For example in Hodgkin´s lymphoma, aggregation of eosinophils together with MCs was described to correlate with poor prognosis (Enblad G et al. 1993). In various tumors including melanoma (Ribatti D et al. 2003), lung cancer (Tomita M et al. 2000) and squamous cell carcinomas of the esophagus (Elpek GÖ et al. 2001) and cervix (Benitez-Bribiesca L et al. 2001), MC infiltrates correlated to microvessel density in addition to tumor progression. In melanoma, immunohistological staining showed increased microvessel density correlating with MC numbers in lesions of poor prognosis compared to those of intermediate and good prognosis. Similar results were obtained for primary cutaneous lymphoma, where increased MC numbers together with increased degranulation correlated with microvessel density and progression (Rabenhorst A et al. 2012). Moreover, several studies using different tumor models observed reduced tumor neo-angiogenesis associated with decreased tumor growth in MC-deficient mice (Gounaris E et al. 2007, Rabenhorst A et al. 2012, Soucek L et al. 2007). Again supporting the concept of a pro-tumorigenic contribution of MCs to tumors, several studies reported a decreased tumor growth upon inhibition of MCs. For example, Gournaris et al. showed, in a murine colon cancer model, that polyps are infiltrated with MCs and depletion of MCs either pharmacologically or through generation of MC-deficient chimeric mice lead to profound remission of polyps (Gounaris et al. 2007). Also, in tumor model of pancreatic islet tumor, activation of Myc triggers tumor expansion associated with recruitment of MCs and pharmacologic inhibition of MC degranulation inhibits tumor growth (Soucek L et al. 2007). MCs were also shown to exert anti-tumorigenic effects in certain malignancies (Tan SY et al. 2005, Amini RM et al. 2007). Furthermore, patients with low MC counts showed more pronounced invasion than those with higher numbers of MCs and, accordingly, the 5-year survival rate was worse in patients with

low MC counts compared to those with high MC counts. Also, in breast cancer, stromal MCs were found to correlate with low grade tumors and a favorable prognosis. Patients with axillary lymph node metastases showed more MCs in the non-involved axillary lymph nodes, indicating a protective effect of MCs in breast cancer (Amini RM et al. 2007). The relationship between MC numbers and tumor invasiveness was recently evaluated in thyroid cancer by our group (Melillo et al., 2010).

MCs are mainly found at the invasive front (the tumor–host interface) of human tumors, where they may facilitate extracellular matrix breakdown and tissue remodeling, and thus promote tumor cell motility. In human PTCs MC infiltrate was present both within the tumors and at the invasive front, but was virtually absent from normal tissues; MC density was increased, to various degrees, in 95% of a group of PTCs (*n*=91) *(Fig. 2)*. Then, the correlation between clinicopathological features, angiogenesis and MC infiltrate has been analyzed. PTCs were divided into two groups: those with intense (+++, ++) and those with weak (+, -) tryptase staining. The group of PTCs with intense tryptase staining, tended to be more invasive, as extrathyroidal extension was more frequent (P=0.0005).

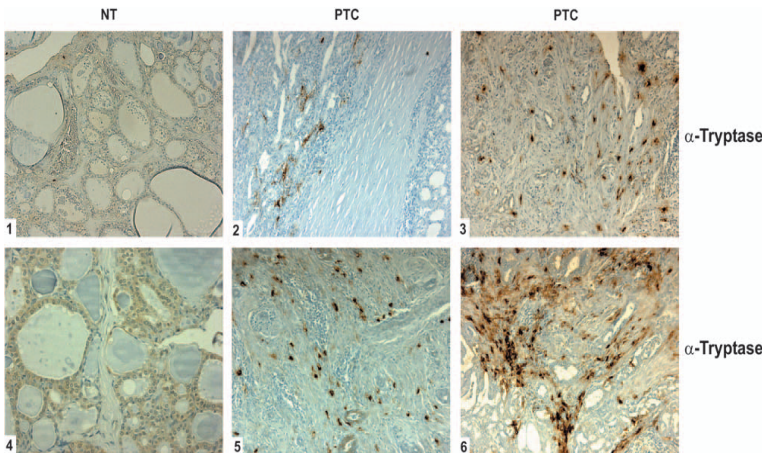

Fig. 2 Immunohistochemical analysis of tryptase in human papillary thyroid carcinoma (PTC). The degree of tryptase-positive cell staining differed among PTC samples (see samples 2,3,5 and 6). Normal thyroid tissues (1,4) were negative for tryptase (Melillo et al. 2010).

Tryptase score	*Clinical features*		P *value*
	Extrathyroidal extension -	Extrathyroidal extension +	
Intense tryptase staining	45% (17/38)	77% (46/58)	**0.0005**
Weak tryptase staining	55% (21/38)	23% (12/58)	

Table 1 Correlation between tryptase score and clinical features of PTCs (Melillo et al. 2010).

In this study, thyroid cancer cells attract MCs through the release of Vascular Endothelial Growth Factor-A (VEGFA). Moreover, human thyroid carcinoma cells, through the release of soluble factors, induce MC degranulation and cytokine synthesis *in vitro*. Consistently, MCs injected in the tail vein of immunodeficient mice are recruited to thyroid carcinoma cell xenografts. Interestingly, when MCs and thyroid cancer cells are co-injected subcutaneously into nude mice, MCs survive and proliferate, suggesting that mediators released in situ can induce these effects. Moreover, when thyroid cancer cells are treated with MC-released mediators they show higher proliferation rate, survival capacity and invasive ability. Accordingly, local co-injection of MCs and thyroid cancer cells accelerate the growth of thyroid carcinoma xenograft in athymic mice. This effect is mediated by increased proliferation and vascularization of thyroid cancer cells and reverted by sodium cromoglycate (Cromolyn), a specific MC-degranulation inhibitor (Melillo et al., 2010). These data suggest that MCs exert a protumorigenic effect on thyroid cancer.

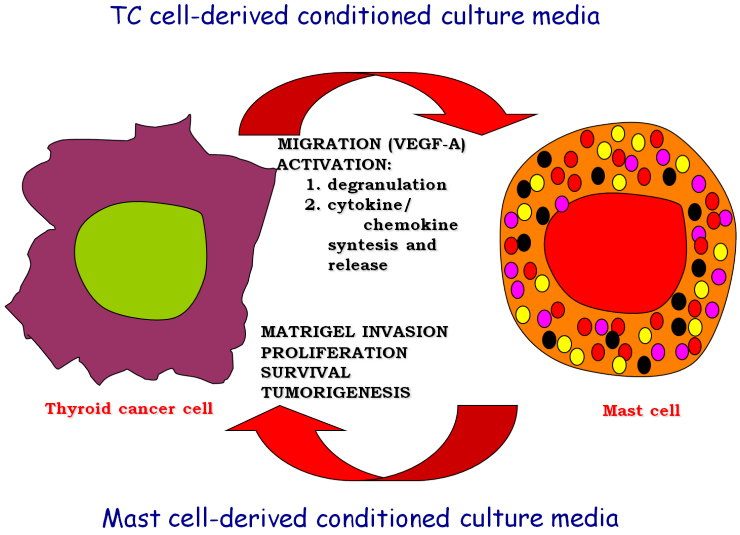

Fig.3 The complex relationship between MC and thyroid cancer cell. MCs exert a protumorigenic effect in thyroid cancer. They are recruited in tumor site by tumor-derived VEGF-A (and possibly by other chemoattractants), and are activated by cancer cell-secreted unknown factors. Activated MCs secrete histamine, and the chemokines CXCL1 and CXCL10, that induce thyroid cancer cell proliferation, survival and invasion (Liotti et al. 2012).

These results indicate that MCs are present in PTCs and that their presence and intensity positively correlate with invasive behavior. The increased motility and invasiveness of tumor cells are reminiscent of epithelial-to mesenchimal transition (EMT) Thus, it is possible that MCs enhance migratory and invasive ability of tumor cells through the induction of EMT.

1.5 Tumor Inflammatory Microenvironment and mediators linking EMT and stemness

1.5.1 The Epithelial to Mesenchymal Transition (EMT)

The Epithelial to Mesenchymal Transition (EMT) is a conserved developmental program whereby a cascade of molecular and physiological changes within a cell results in the transformation of the epithelial cell into a phenotypically and genetically distinct mesenchymal cell. During EMT, epithelial cells lose the adherents junctions that keep them in contact with their neighbors. They gain a mesenchymal cell phenotype that enables them to break through the basal membrane and migrate over a long distance, a result of profound changes in their cytoskeleton architecture and gene expression profile (Kalluri and Neilson 2003) *(Fig. 4)*. However, this "transformation" is reversible: mesenchymal cells can revert back to epithelial cells through a reverse process called mesenchymal-epithelial transition (MET). EMT does not only occur during embryonic development or as a physiological response to injury. It is also an important element in cancer progression and other pathologies that involve organ degeneration, such as fibrosis. At the cellular level, pathological EMTs are very similar to physiological EMTs in that they are governed by similar signaling pathways, regulators, and effective molecules. From a clinical perspective, metastasis is the most critical aspect of tumorigenesis (Hanahan and Weinberg 2011). However, additional steps must be completed before a metastatic tumor is successfully established. The spread of malignant cells consists of a series of steps, all of which are thought to be important for metastatic outgrowth in different organs. Basically, these steps include local invasion toward and entry into blood vasculature (intravasation), survival within the circulation system, arrest in distant capillary beds or “homing” to distal organs, exit from blood vasculature (extravasation), and eventual outgrowth and re-establishment of malignant growths in secondary locations (Fidler 2003; Hanahan and Weinberg 2011).

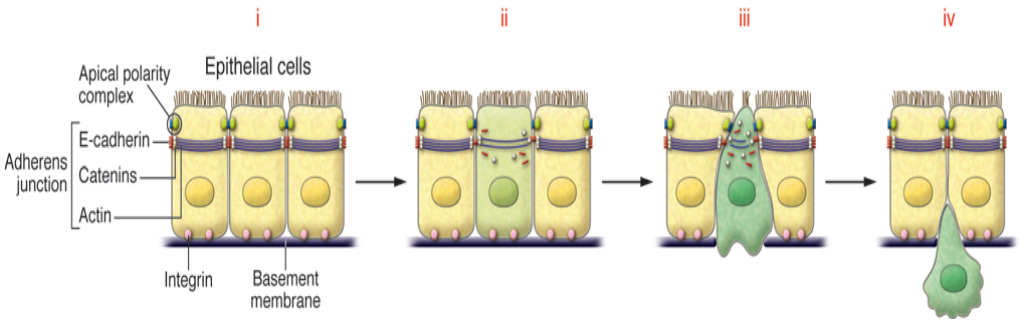

Fig. 4 In epithelial cells, E-cadherin and integrins provide cell-cell and cell-basement adhesion, respectively (i). During EMT, the transcription of E-cadherin and integrins in epithelial cells is repressed, inducing the loss of polarity in these cells (ii). To invade the underlying tissue, the basement membrane is disrupted and the cytoskeleton remodeled to induce a migratory morphology (iii). This allows the cells to detach from the epithelial sheet and move into the surrounding stroma (iv) (Modified from Acloque et al., 2009).

1.5.2 Characterization of EMTs

EMTs are categorized into three separate types based on the context in which they occur. Type 1, 2 and 3 EMTs can have different outcomes within a given cellular context, however it is currently not clear what biochemical elements they share and how they differ.

Type 1 EMTs occur during development and are utilized during discrete morphogenic events. For example, the EMT program is activated during gastrulation in a distinct population of epiblast cells. Upregulation of transcription factors such as SNAIL1 leads to MMP expression facilitating breakdown of the underlying basement membrane and ingression through the primitive streak. These mesendodermal cells then either undergo reversion of EMT (termed MET) and form the ectoderm or retain their mesenchymal properties and give rise to the mesodermal layer. During neurulation, populations of migratory cells termed neural crest cells are formed at the neuroectoderm-ectoderm junction. Activation of EMT facilitates their delamination and subsequent migration throughout the developing embryo where these cells give rise to various tissues.

Type 2 EMTs are associated with inflammation and involve the conversion of epithelial cells to mesenchymal cells in response to physiologic stress and are critical to tissue repair and regeneration. The re-epithelization stage during wound-healing involves drastic changes in the morphology and function of surrounding keratinocytes. During wound closure, keratinocytes activate a partial and reversible EMT to decrease intercellular adhesion and increase their cell-matrix interaction to migrate over and close a lesion. Type 2 EMT can also be activated in tissues undergoing pathologic stress. For example, liver or kidney damage can lead to the formation of fibroblasts from existing epithelial cells to promote tissue repair. Although type 2 EMTs normally cease once

inflammation has subsided, persistent inflammation can lead to constitutive activation of EMT and tissue or organ damage.

Type 3 EMTs are specific to neoplastic epithelial cells that have undergone genetic changes including upregulation of oncogenes and decreased expression of tumor suppressor genes. The activation of EMT in cancer cells can render them far more invasive and malignant than those cells undergoing type 1 and 2 EMTs. This is possibly due to their ability evade the normal checkpoints and controls of the body. Increasing evidence suggests that the metastatic spread of cancer cells is facilitated through activation of EMT in a subset of tumor cells during cancer progression. The absence of E-cadherin in tumor samples has long been correlated with increased tumor grade and poor prognosis and upregulation of numerous EMT markers is also correlated with decreased patient survival (Gomez, I et al 2011, Kosaka, T et al. 2010). Additionally, cancer cells at invasive fronts can take on an EMT-like phenotype (Spaderna S et al. 2006)

1.5.3 Molecular regulation of EMT

The hallmark of EMT is the loss of E-cadherin expression, an important caretaker of the epithelial phenotype. Loss of E-cadherin expression is often correlated with the tumor grade and stage, because it results in disruption of the cell-cell adhesion and an increase in nuclear beta-catenin (Cowin, Rowlands et al. 2005; Junghans, Haas et al. 2005). Several transcription factors have been implicated in the regulation of EMT, including zinc finger proteins of the SNAIL/SLUG family, the basic helix-loop-helix factors Twist1/2, E12/E47, Goosecoid, ZEB1and SIP1 (Nieto 2002; Yang, Mani et al. 2004; Hartwell, Muir et al. 2006).
Beside directly affecting gene expression, the zinc finger proteins of the SNAIL and ZEB family and the basic helix-loop-helix transcription factor Twist1/2 reprogram gene expression by complex interaction with other transcription factors and pathways and furthermore establish interconnected feedback loops that sustain the morphological transition. The impact of SNAIL, SLUG, TWIST and ZEB transcription factors on cell morphology is based on a common mechanism: all of them have been reported to bind to E-box motifs of various promoters either transcriptionally repressing or activating the downstream gene (Peinado et al. 2007; Vesuna et al. 2008; Mironchik et al. 2005). EMT transcription factors have been shown to equip the cell with migratory capacity by activating especially mesenchymal genes, including those encoding for N-cadherin, the extracellular matrix component Fibronectin and cytoskeletal proteins such as Vimentin and Smooth Muscle Actin (alpha-SMA) (LaGamba, Nawshad, and Hay 2005). In contrast, the repressive functions of the major EMT transcription factors are much better characterized.

In order to abrogate epithelial cell morphology, which is characterized by strong cell adhesion allowing the formation of a dense but permeable polarized layer of cells, EMT transcription factors repress a specific set of cytoskeletal, junctional and other epithelial genes (*Fig 5).*

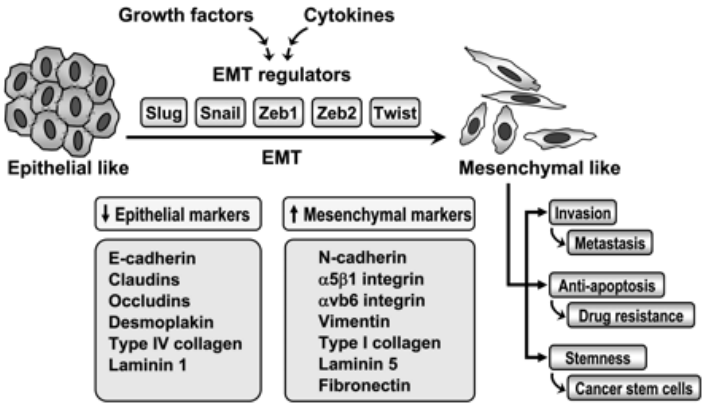

Fig. 5 The EMT regulators transform the cancer cells from epithelial-cell like to mesenchymal-cell like with suppression of epithelial markers and expression of mesenchymal markers. The final effects on cancer cells are cancer metastasis, drug resistance and with features of cancer stem cells. Modified from Jin-Yuan Shih and Pan-Chyr Yang, 2011.

Aberrant activation of the EMT program can be hazardous to cellular and tissue homeostasis. For this reason, the transition from epithelial to mesenchymal cell is orchestrated via an intricate and complex set of internal and external signals. The TGF-β proteins are well-characterized, potent inducers of EMT. After injury, metalloproteinases (MMPs) secreted by stromal cells surrounding a wound can activate TGF-β stored in the extracellular matrix and stimulate activation of a temporary and reversible EMT in keratinocytes allowing their migration into the wound site (Savagner P et al. 2005). TGF-β signals can synergize with other major EMT signaling pathways to induce EMT. The Notch signaling pathway has been shown to be a downstream mediator TGF-β induced EMT. In mammary epithelial cells, TGF-β activation induces the expression of the Notch ligand Jagged1 (Jag1) and EMT progression. siRNA against Jag1 inhibits EMT despite TGF-β expression (Zavadil J et al 2004). The Wnt pathway is also involved in EMT activation. Neural crest induction in the chick requires Wnt1-mediated activation of SLUG (SNAIL2) and expression of a dominant-negative Wnt1 inhibits SLUG transcription and neural crest migration. Twist1 is also induced by Wnt1 in mammary ephitelial cells and its promoter is responsive to β-catenin (Howe L.R. 2003). Although there are limited studies connecting Hedgehog signaling and EMT, its pharmacologic inhibition in the E3LZ10.7 pancreatic cancer cell lines restores expression of E-cadherin and decreases SNAIL expression and

metastatic potential (Feldmann G et al. 2007). Additionally, the Hedgehog effector Gli can induce SNAIL1 mRNA in RK3E rat kidney cells and results in nuclear translocation of β-catenin and cell transformation (Li X et al. 2006). The Src family of tyrosine kinases are found at the inner layer of the plasma membrane of cells and localize with adherens junctions as well as cell matrix integrin; elevated Src activity at cell-cell junctions leads to phosphorylation of E-cadherin and β-catenin, E-cadherin endocytosis, adherens junction disassembly and EMT. Src effects on cell-cell junction is observed during would healing where Src is activated to promote cell migration and wound closure. Receptor tyrosine kinases (RTKs) are a major group of cell-surface receptors and many of their ligands are inducers of EMT. Epidermal Growth Factor (EGF) secreted by stromal macrophages can promote EMTs in adjacent tumor cells. These cells, in turn, respond to macrophage-derived EGF signaling by secreting CSF-1, a macrophage attractant and stimulant (Weinberg R.A. 2007) thereby perpetuating EMT activation. Hepatocyte growth factor (HGF) expression in epithelial cells induces SNAIL1 expression and cell scattering (Grotegut R et al. 2006). Platelet-derived growth factor (PDGF) overexpression in prostate cancer cells leads to E-cadherin downregulation via mTOR . Fibroblast growth factor (FGF) and IGF (Insulin-like growth factor) (Morali OG et al. 2001, Billottet et al. 2004)growth factors have also been implicated in mediating EMT. Ras is a major effector of RTK-mediated EMT (Edme E et al. 2002) and acts through the MAPK pathway to activate EMT transcription factors such as SNAIL1, SLUG and Twist1 (Wang Z et al. 2007).

1.5.4 EMT and STEMNESS

A large body of research has described stem cells in normal tissues, which are capable of renewing themselves through asymmetrical cell division while simultaneously generating committed progenitor cells whose descendants may eventually differentiate and carry out tissue specific functions (Reya et al., 2001). Recent evidences support the hypothesis that tumors contain a subpopulation of tumor cells called cancer stem cells (CSCs), which exhibit stem-like cell properties such as the ability to self-renew, form tumor spheres in low adherence conditions, differentiate into various populations of cancer cells, and form new tumors in a xenotransplant system (Dontu et al., 2003; Gupta et al., 2009). While some studies suggest that CSC may arise from the transformation of their normal counterparts, recent observations suggest that they originate from fully differentiated cells through an adaptive transdifferentiation program *(Fig. 6)*.

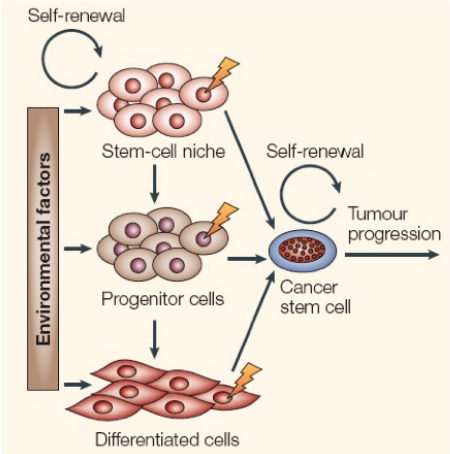

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Fig. 6 The "cancer stem-cell theory" is based on the assumption that during tissue regeneration, the amplification of progenitor cells opens a window of time suitable for accumulating genetic alterations, leading to the emergence of cancer cell-stems (CSCs). CSCs would thus initiate and sustain tumour growth. Alternatively, under stress conditions, fully differentiated cells reacquire stem-like properties, including self-renewal properties. This gain of function is influenced by microenvironmental conditions. These cells could potentially be prone to transformation and give rise to CSCs.

CSCs were first identified in the hematopoietic system (Bonnet and Dick, 1997); more recently, however, they have also been discovered in solid tumors, including those arising in the breast, colon, brain, glioma, melanoma, prostate, lung, ovarian, renal cell and thyroid carcinomas (Al-Hajj et al., 2003; O'Brien et al., 2007; Ricci-Vitiani et al., 2007; Singh et al., 2004, Todaro M et al. 2010). During the process of tumor metastasis, which is often enabled by EMTs (Thiery et al. 2003), disseminated cancer cells would seem to require self-renewal capability, similar to that exhibited by stem cells, in order to spawn macroscopic metastases. This raises the possibility that the EMT process, which enables cancer cell dissemination, may also impart a self-renewal capability to disseminating cancer cells. Indeed, the metastatic process is at least superficially similar to the processes that occur during tissue repair and regeneration and enable adult stem cells to exit tissue reservoirs (such as the bone marrow), enter and survive in the circulation, and exit into secondary tissue sites, where they proliferate, differentiate, and participate in tissue reconstruction (Kondo et al., 2003).
Together, these diverse lines of evidence suggested a link between cancer stem cells and the mesenchymal-appearing cells generated by EMTs *(Fig.7*) (Polyak and Weinberg 2009)

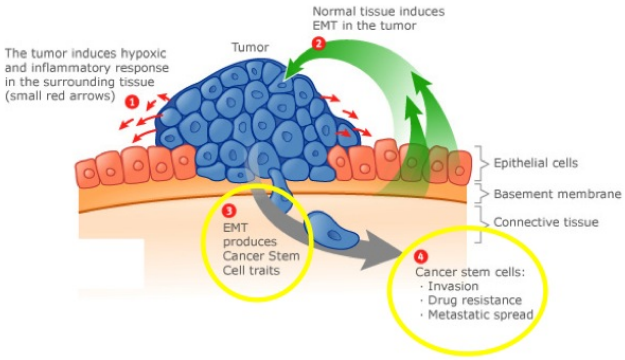


Fig. 7 Cancer stem cells may be result of the induction of EMT in more differentiated cancer cells. (Modified from http://www.bergenbio.com/science_of_emt)

The CSC is thought to share several key features with normal stem cells: unlimited capacity for self renewal, including maintenance of the CSC population through asymmetric division, the ability to differentiate into several cell lineages and intrinsic resistance against cytotoxic therapies through drug-efflux mechanisms and slow cell cycling. The identification and analysis of CSCs are fundamental for cancer study. The "gold standard" to identify CSCs is to isolate cells using biomarkers (cell surface marker), to test their sphere-forming ability in low-adherence condition and tumorigenicity at serial passaging, that is, CSCs derived from primary xenograft tumors should initiate tumor growth and give rise to secondary tumors. Regarding biomarkers, recent studies have reported several CSC markers in solid tumors (e.g. CD44+/CD24- for breast cancer, CD133+ for brain tumor and colon cancer, CD44+ for gastric cancer). In some types of cancers, two or more different markers have independently been identified in the same type of cancer: for example, CD44+/CD24-, aldehyde dehydrogenase 1 (ALDH) activity, and CD133 for breast CSCs; CD133 and CD44+/CD24+ for colon CSCs. However, their association and/or combination have not been fully investigated.

There are several canonical markers that CSCs shared with normal stem cell: SOX2 (SRY-related HMG-box gene 2), initially reported to be linked strongly with the inhibition of neuronal differentiation, has been shown to acts as an important transcriptional factor to maintain the self-renewal capability of embryonic stem cell (ESCs). OCT4 (OCT3/4), a member of the family of POU domain transcription factor known to bind SOX2, is also the key regulator essential for the pluripotency and self-renewal of of ESCs. Nanog, a homeodomain-containing protein, maintains pluripotency of mouse ESCs by inhibiting NFκB and cooperating with Stat3. ABCG2 is a member of the ATP binding cassette (ABC) transporters, which can pump a wide variety of endogenous and exogenous compounds out of cells. Widely expressed in stem cells, ABCG2 is also found to confer the side population phenotype and is

recognized as a universal marker of stem cells; Nestin is an intermediate filament protein that is known as a neural stem/progenitor cell marker.

Increased aldehyde dehydrogenase (ALDH) is a hallmark of cancer stem cells measurable by the aldefluor assay. Cytosolic aldehyde dehydrogenases (ALDHs) are a group of enzymes involved in oxidizing a wide variety of intracellular aldehydes into their corresponding carboxylic acids. ALDH1, one of 19 ALDH isoforms expressed in humans, was a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome of breast cancer patients. High ALDH1 activity has been used to define stem cell populations in many cancer types has been described in primitive cells from multiple myeloma, acute myeloid leukemia, pancreatic, breast, and lung carcinomas (Lohberger et al. 2012).

Recent progress in thyroid cancer biology has revealed that the histological appearance and biological behaviors of thyroid carcinoma often show heterogeneity, with cells exhibiting distinct proliferative and differential capacities (Pilotti S et al. 1995, Aratake Y et al. 2006). Emerging evidence indicates that a rare subpopulation of cells appeared to be cancer stem cells (CSCs) drive thyroid cancer heterogeneity and contribute to the resistance to cancer therapy as observed in various cases (Ke CC et al. 2013, Zheng X et al. 2010, Todaro M et al. 2010). Moreover, clinical observations have revealed that CSC marker frequency in thyroid cancer is related to adverse outcomes (Yun JY et. Al 2013). Thus, CSCs have been indicated to play a crucial role in the malignant progression and therapeutic resistance of thyroid cancer (Zhu W et al. 2010). Lan *et al* and Yasui *et al* as well as other research groups observed that more differentiated thyroid cancer cell populations acquire CSC properties through EMT (Lan L et al.2013, Yasui K et al. 2013). These studies, therefore, raise the possibility that thyroid CSCs may arise from restricted progenitors or more differentiated cells that have acquired self-renewing capacity. Compared with other organs' cancer, studies of thyroid CSCs are still limited, and further researches are definitely required, although ALDH is a major marker to enrich thyroid CSCs (Todaro M et al. 2010, Mika Shimamura et al. 2014).

The CSC hypothesis has fundamental and important clinical implications, as the current development of cancer therapeutics is largely based on screening agents with the ability to cause bulk tumor, but not CSC, regression in animal models or in clinical trials (Bao *et al*., 2006; Bao *et al*., 2008; Gupta et al., 2009). Strategies aimed at efficiently targeting CSCs are critical for monitoring the progress of cancer treatment and for evaluating new therapeutic agents. The elucidation of signaling pathways which regulate CSC self-renewal and survival provides potential therapeutic targets. In addition, CSC behaviors are constantly regulated both by inside regulators such as transcription factors and external signals from their niches, including neighboring stromal, immune, and non-stem tumor cells. Targeting the neighboring non-stem cancer cells, stromal cells or the paracrine factors secreted by these cells may target CSCs indirectly, thereby contribute to long-term remissions and treat carcinoma resistant to current therapies (Polyak and Hahn, 2006).

1.5.5 The role of inflammatory cells and cytokines in EMT and stemness

EMT and stemness may be influenced by tumor microenviroment (Joyce and Pollard 2009), which is composed by multiple cell types, such as MC (MC), fibroblasts, epithelial cells, macrophages, myeloid-derived suppressor cells (MDSCs). Stromal cells are recruited to tumors, not only to enhance growth of the primary tumor, but also to facilitate its metastatic dissemination to distant organs (Tse and Kalluri 2007; Lunt, Chaudary et al. 2009). EMT commonly occurs at the invasive front (tumor-stromal boundary) of many invasive carcinomas (Christofori 2006; Franci, Takkunen et al. 2006) and is triggered by cellular signals from microenvironment. Tumor stroma conteins various cell types such as cancer associated fibroblasts (CAFs), mesenchymal stem cells (MSCs) and immune cells (Joyce and Pollard al. 2009, Lin EY et al. 2004, Tse JC and Kalluri R 2007,); CAFs, MSCs and Th2 polarized CD4+/CD8+ T-lymphocytes have been shown to contribute to EMT at the tumor-stroma interface (Santisteban M et al. 2009). Tumor associated macrophage (TAM), the major inflammatory component of the stroma in malignancies, promote proliferation, tissue remodelling, extracellular matrix, invasion and matrix breackdown through protease production and release of a variety of growth factor (Condeelis and Pollard 2006). TAM have likewise been shown to induce EMT at the invasive front mainly through TNF-α mediated stabilization of SNAIL, a key mediator and marker of EMT (Bates RC et al. 2003). TAMs induced EMT in tumor cells located at the invasive front correlates with metastatic disease in a murine breast cancer model. Several studies indicate that, once EMT is initiated, the permanence of tumor cells in a mesenchymal status post-EMT is dependent on the existence of autocrine cytokine and/or growth factor loops that were initially responsible for the induction of EMT in the same cells. Induction of tumor EMT was shown to be enhanced by the secretion of a subset of inflammatory cytokines, various chemokines and angiogenic factors, which included TGF-β. TGF-β is a multifunctional cytokine regarded as being a tumor suppressor in normal epithelial cells and during early tumor growth, while acting as a potent tumor-promoting factor in late-stage tumors (Zavadil J et al 2005). In late-stage or metastatic tumors, TGF-β promotes angiogenesis, recruits various cell types to the site of the tumor, including fibroblasts and immune cells, suppresses a functional anti-tumor immune response and induces tumor cell migration, invasion and EMT (Zavadil J et al 2005). In multiple tumor cell models, it has also been demonstrated that, once tumor cells have undergone EMT, secretion of TGF-β is markedly upregulated (Kudo-Saito C et al 2009). Once secreted, TGF-β has the ability to function in an autocrine fashion, contributing to the maintenance of the mesenchymal and stem cell phenotypes of carcinomas that have passed through EMT.

Similar results were observed with the secretion of the cytokine IL-6 (Sullivan NJ et al 2009) or VEGF (Gonzalez-Moreno O et al. 2009) by tumor cells undergoing EMT. IL-6 is a pleiotropic cytokine involved in the differentiation and growth of hematopoietic stem cells, T cells and B cells, as well as the differentiation of other cell types that express IL-6R (CD126) and the coreceptor gp130 (CD130). IL-6 is a well-known regulator of immune responses and it has also been implicated as a potent growth factor for certain tumor cell types, including breast cancer cells. Recent reports also demonstrate a role for IL-6 in tumor EMT and in the acquisition of stemness. Breast cancer cells overexpressing Twist, for example, have been shown to upregulate the secretion of IL-6 and, at the same time, to activate STAT3, indicating the existence of a positive feedback loop involving autocrine-mediated IL-6 signaling events (Sullivan NJ et al. 2009).

2. AIM OF THE STUDY

MCs (MC) play an important role in the innate and adaptive immune responses but, recently, their involvement in tumor growth, angiogenesis and metastasis has been described. Previous data indicate that MC are mainly found at the invasive front (the tumor-host interface) of human PTCs and the intensity of such infiltrate correlates with capsule invasion, suggesting a role for these cells in tissue invasion.

With this study, we propose:

• to identify the major actor(s) among MC-derived mediators that could recapitulate EMT induction, in order to employ a direct-target strategy for the treatment of thyroid cancer;

• to evaluate whether MC-induced EMT occurs together with increased stemness of thyroid cancer cells;

• to evaluate whether the density of MC infiltrate correlates with increased EMT and stemness in thyroid cancer surgical samples.

3.MATERIALS AND METHODS

3.1 Cell cultures, transfection and Sphere Forming assay

Nthy-ori (or Nthy-ori 3.1) are human SV-40 immortalized thyroid epithelial cells. Human thyroid papillary cancer cell lines TPC1 harbor a RET/PTC1 rearrangement; the anaplastic cells 8505-C harbor a BRAF (V600E) mutation in homozygosis (Salvatore et al. 2006). All these cell lines were maintained in DMEM (Dulbecco's Modified Eagle Medium), which contains 4.5g/L of LD-glucose and 0.11g/L of sodium pyruvate, supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin and 1% glutamine (Life Technologies Inc., Pasley, PA); they were kept in incubator at 37˚C in humidified atmosphere containing 95% air and 5% CO_2. HMC-1 (Human MC Line 1) were kindly donated by JH Butterfield and grown in Iscove's (Life Technologies) supplemented with 10% fetal calf serum without α- thioglycerol (Butterfield et al., 1988). LAD-2 cells (kindly donated by AS Kirshenbaum) were grown in StemPro-34 (Life Technologies) supplemented with human recombinant Stem Cell Factor (100 ng/ml) (Peprotech, Rocky Hill, NY).

For isolation of human lung MCs (HLMCs), lung tissue was obtained from patients undergoing thoracotomy and lung resection. Macroscopically normal parenchyma was dissected free from pleura, bronchi, and blood vessels and minced into a single-cell suspension as previously described (de Paulis et al. 2000). Yields with this technique ranged between 3×10^6 and 18×10^6 MCs, and purities were between 1 and 8%. Lung MCs were purified by countercurrent elutriation (J2/21; Beckman) and then by discontinuous Percoll density gradient as previously described (de Paui et al. 2000). MCs were further purified to near homogeneity by positive selection using an antibody anti-CD117 conjugated to magnetic beads and retaining labeled cells on a selection column (MACS system; Miltenyi Biotec, Bergisch Gladbach, Germany). The final preparations contained > 95% viable cells, assessed by the Trypan blue exclusion method, and purity was > 98% MCs.

For the spheroid-forming assay, single cells were plated at 15 cells/ well on ultra-low-attachment 96-well or 1500 cells in 100mm plates (Corning) in serum-free Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 Ham (DMEM/F12 = 1:1, Life Technologies), supplemented with 2% B27 and enriched for 10 ng/ml of EGF (*epidermal growth factor*) (Miltenyi Biotec) and 20 ng/ml of bFGF (*basic fibroblast growth factor*) (Miltenyi Biotec) growth factors. Cells were maintained in a humidified atmosphere of 5% CO2 and 95% air at 37°C. Spheres were counted after 15 days. For in vitro serial passaging, thyrospheres were collected by gentle centrifugation at 800 rpm for 5 min and dissociated mechancally. The dissociated cells were passed through 40 mm mesh filters (BD Falcon Cell Strainer, Franklin Lakes, NJ) to eliminate

doublets or triplets. Single cells were plated at 1500 cells/well on ultra-low-attachment six-well plates to generate secondand third generations of thyrospheres or 1 cell/well for Limiting Dilution Assay.

3.2 Protein studies

Protein extractions and immunoblotting experiments were performed according to standard procedures. Briefly, cells were harvested in lysis buffer (50 mM HEPES, pH7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EGTA, 1.5 mM MgCl2, 10 mM NaF, 10 mM sodium pyrophosphate, 1 mM Na3VO4, 10 µg of aprotinin/ml, 10 µg of leupeptin/ml) and clarified by centrifugation at 10,000 x g for 30 min. Protein concentration was estimated with a modified Bradford assay (Bio-Rad, Munich, Germany). Immune complexes were detected with the enhanced chemiluminescence kit (ECL, Amersham).

3.3 Generation of stable clones

850-5C cells at approximately 70% confluence were stably transfected with pBabe-puro empty vector or co-transfected with pBabe-puro and pCMV6-XL5 IL-8 vector (OriGene) by electoporation method (Neon® Transfection System for Electroporation, life technologies). Stably transfected cells were selected adding puromycin (500 ng/ml) to culture dishes two days after transfection. Puromycin-resistant colonies were then harvested and analyzed for protein expression and biological activity.

TPC-1 were tranfected with SLUG Plasmid (#31698-Addgene) or with empty vector by electoporation method (Neon® Transfection System for Electroporation, life technologies). Stably transfected cells were selected adding G418 (Life technologies) (1.2 mg/ml) to culture dishes two days after transfection. G418 -resistant colonies were then harvested and analyzed for protein expression and biological activity.

3.4 Antibodies, cytokines and drugs

Anti-ZEB-1, anti-SLUG, anti-SNAIL, anti-phospho-AKT (specific for the active AKT phosphorylated at Serine 473), anti-phospho-MEK1/2 (specific for the active MEK1/2 phosphorylated at Serine 217/221) and anti-phospho-STAT3 (specific for the active STAT3 phosphorylated at Tyrosine 705) for Western Blot analysis were from Cell Signaling Technology (Beverly, MA). Monoclonal anti-tubulin was from Sigma Chemical Co. Secondary anti-mouse and anti-rabbit (Biorad) antibodies are coupled to horseradish peroxidase. Anti-Claudin-1 for Immunofluorescence analysis and for Facs analysis was from Acris Antibodies (Germany), PanCytokeratin Ab from Sigma Aldrich. Human CXCL8/IL-8 Mab (Clone 6217), Human CXCR1/IL-8 RA Mab (Clone 42705),

Human CXCR2/IL8 RB (Clone 48311) were from R&D; HA-probe (Y-11) SC-805 from Santa Cruz. α-IL6, α-IL8 and α-TNFα for immunodepletion were from BD Biosciences. Recombinant human TGF-β, IL-6, IL-8 and TNFα were from Peprotech. S31-201-STA3 inhinitor-(Selleck), LY-294002-AKT inhibitor-(Cell Signaling Technology), U0126-MAPK inhibitor-(Cell Signaling Technology) and Cromolyn sodium salt -Mast cel inhibitor-(Sigma-Aldrich).

3.5 RNA, cDNA and Q-PCR

Total RNA was isolated by the RNeasy Kit (Qiagen Hilden, Germany); the principle of this purification is the affinity chromatography during which RNA longer than 200 bases binds to the RNeasy silica membrane. Cells were harvested in lysis buffer RTL supplemented with β-mercaptoethanol (10 µl per 1 ml of lysis buffer). After the addition of ethanol 70%, the lysate was loaded on the column and centrifuged. Then, the washing buffer RW1 was added into the column which was centrifuged again and, consequently, it has been possible to carry out the treatment with DNase, dissolved in buffer RDD, to eliminate possible contaminations due to the presence of total genomic DNA. Other washes were performed with RW1 and RPE buffers; RNA, bound to the affinity column's resin, was eluted by adding sterile H_2O RNase free (DEPC water), to avoid degradation. It is possible to proceed with the cDNA synthesis. Random-primed first strand cDNA were synthesized in a 50 µl reaction volume starting from 2 µg RNA using the GeneAmp RNA PCR Core Kit (Applied Biosystems, Warrington, UK). RNA was incubated with $MgCl_2$, 10x buffer solution, Random Hexamers (RH), RNase Inhibitor (RI), dNTP (Deoxyribonucleotide triphosphate) and Reverse Transcriptase (RT). To start the cDNA synthesis, the enzyme uses an RNA-complementary oligonucleotide; the synthesized single-strand DNA is then used as template for the synthesis of the second strand and then for the amplification with PCR. RNase (usually derived from the bacterium Escherichia coli) degrades the original RNA strand that has been used as a template by reverse transcriptase. Thus, the mechanism consists of repeated cycles of amplification: denaturation of DNA template, primers-annealing to the target sequence (which occurs at the annealing temperature dependent on their length and base-composition) and a final extension.

Q-PCR allows to measure the levels of a gene expression after its cDNA amplification; a specific fluorescent dye, SYBR Green, which intercalates in the double-strand DNA, is used to quantize amplified cDNA and the increase in fluorescence is directly proportional to the amount of amplified sample present in the reaction. This method was performed by using the SYBR Green PCR Master mix (Applied Biosystems) in the iCycler apparatus (Bio-Rad, Munich, Germany). Amplification reactions (25 µl final reaction volume) contained 200 nM of each primer, 3 mM $MgCl_2$, 300 µM dNTPs, 1x SYBR

Green PCR buffer, 0.1U/ µl AmpliTaq Gold DNA Polymerase (Applied Biosystems), 0.01U/ µl Amp Erase (Applied Biosystems), RNase-free water and 2 µl cDNA samples. Thermal cycling conditions were optimized for each primers pair. To verify the absence of non-specific products, 80 cycles of melting curve (55°C for 10 sec) were performed. As previously described, amplification is monitored by measuring the increase in fluoresce caused by the SYBR-Green binding to double-strand DNA and the amplification of GAPDH (Glyceraldehyde 3-phosphate dehydrogenase: housekeeping gene, constitutively expressed) was used for normalization. Fluorescent threshold values were measured in triplicate and fold changes were calculated by the formula $2^{-(\text{sample 1 } \Delta Ct \text{ - sample 2 } \Delta Ct)}$, where *ΔCt* is the difference between the amplification fluorescent thresholds of the mRNA of interest and the GAPDH mRNA. Primers were designed by using software available at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi and synthesized by CEINGE (Naples, Italy).

The employed primer sequences are reported below:

hSOX2	**F** 5'-gcgaaccatctctgtggtct-3'
	R 5'-aaaatggaaagttgggatcg-3'
hNANOG	**F** 5'-tacctcagcctccagcagat-3'
	R 5'-ttgctattcttcggccagtt-3'
hABCG2	**F** 5'-tggatttacggctttgcagc-3'
	R 5'-tcctgttgcattgagtcctgg-3'
hOCT4	**F** 5'-agcaaaacccggaggagt-3'
	R 5'-ccacatcggcctgtgtatatc-3'

hALDH	**F** 5'-gcaactgaggaggagctctg-3'
	R 5'-ttcgattaaatcagccaacttgt-3'
hSLUG	**F** 5’-ccttcctggtcaagaagcat-3’
	R 5’-cacagtgatggggctgtatg-3’
hGAPDH	**F** 5’-ctgccactgaaaaggaggag-3’
	R 5’-ttggcactccttgggttatc-3’
hZEB-1	**F** 5’-aactgctgggaggatgacac-3’
	R 5’-gtcctcttcaggtgcctcag-3’
h SNAIL	**F** 5’-ctctaggccctggctgctac-3’
	R 5’-gcctggcactggtacttctt-3’
hVimentin	**F** 5’-gtttcccctaaaccgctagg-3’
	R 5’-gacacggacctggtggac-3’
h E-Cadherin	**F** 5’-tgcccagaaaatgaaaaagg-3’
	R 5’-gtgtatgtggcaatgcgttc-3’
hMMP3	**F** 5’-tgctttgtcctttgatgttg-3’
	R 5’-ggaagagatggccaaaatga-3’

hMMP10	**F**	5’-gaggaggactccaacaagga-3’
	R	5’-tccaacccaaggaacttctg-3’
hMMP12	**F**	5’-cgtggaggtcatggagactt-3’
	R	5’-ttcgtcctcatcgaaatgtg-3’

3.6 Wound Healing

The Wound Healing assay is a simple and inexpensive method used to estimate the migration and proliferation rates of different cells and culture conditions in vitro mimicking cell migration during wound healing in vivo. The basic steps involve creating a “wound” in a cell monolayer, capturing the images at the beginning and at regular intervals during cell migration in close the wound and comparing the pictures with a positive and/or negative control to estimate the migration rate of the cells. Confluent monolayers of TC cells were treated with mitomycin (2μg/ml for 2 h) (Sigma-Aldrich, USA). Wounded monolayers were incubated for 12 or 24 h at 37°C. Migration was assessed by counting the number of cells that crossed the wound border.

3.7 Immunofluorescence

Samples were fixed with 100% ethanol for 30 minutes at +4°C and with acetone for 3 minutes at -20°C and then washed twice with PBS1x. Subsequently, cells were incubated in 1% BSA-PBS1x solution for 20 minutes at RT (Room Temperature) to saturate non-specific binding sites and washed three times with PBS1x. The primary antibodies, dissolved at working concentration in 0,2% BSA-PBS1x solution, was applied and cells were incubated for 1 hour at 37°C in humidified atmosphere. After three washes, samples were incubated for 45 minutes at 37°C in humidified atmosphere with the secondary antibody, and dissolved 1:50 in 0,2% BSA-PBS1x. After three washes, cells were incubated for 20 minutes at RT in dark humidified atmosphere with DRAQ5 (a red nuclear dye with high affinity for double – stranded DNA, dissolved 1:1000 in PBS1x). Cells were also stained with Hoechst (a blue fluorescent dye used to stain DNA and dissolved 1:8000 in PBS1x) and incubated for 5 minutes at RT. After the last two washes, coverslips were mounted with a drop of Mounting Medium (3μl, glycerol/PBS1x 1:1) and cells were visualized using Zeiss LSM (Laser Scanning Microscope) 510 META confocal microscope.

3.8 ELISA assay

Thyroid cells plated in 6-well dishes were allowed to grow to 70% confluency and then serum-deprived for 24 h. Culture media were cleared by centrifugation at 2,000 RPM at 4°C to remove detached cells and debris. IL-8, TNF-α and IL-6 levels in culture supernatants were measured using a quantitative immunoassay ELISA kit (QuantiKine colorimetric Sandwich assay ELISA, R&D Systems, UK), following the manufacturer's instructions. Triplicated samples were analyzed at 490 nM with an ELISA reader (Model 550 microplate reader, Bio-Rad).

3.8 FACS Analysis

Briefly, cells were incubated (30 min at 4°C) with specific or isotype control Abs. Cells were analyzed with a FACS Calibur cytofluorimeter using CellQuest software (BD Biosciences, Canada). A total of 10^4 events for each sample were acquired in all cytofluorimetric analyses. When necessary, we performed a cell membrane permeabilization using the Cytofix/Cytoperm kit (BD Biosciences, Canada).
The ALDEFLUOR assay (Stemcell Technologies) was performed according to the manufacturer's guidelines to identify cells with high ALDH activity. Cells were suspended in ALDEFLUOR assay buffer (~500,000 cells/ml) plus BODIPY aminoacetaldehyde (BAAA), a fluorescent non-toxic substrate for ALDH which freely diffuses into intact and viable cells, and under the enzyme activity is converted into a metabolite, BODIPY-aminoacate (BAA), which is retained inside the cells. Cells were incubated for 45 minutes at 37°C. As a negative control, to estimate background fluorescence, an aliquot of cells from each sample was treated with 50 mmol/L of the ALDH inhibitor diethylaminobenzaldehyde (DEAB). Supernatants were removed by centrifugation and cell pellets were resuspended in ALDEFLUOR Assay. Buffer to inhibit active efflux of the reaction product. Intracellular fluorescent product was measured by flow cytometry and sorting gates were established using the negative controls

3.9 Xenografts in Mice

Each group of 10 mice (4-week-old male CD1a nu/nu) was inoculated subcutaneously into the right dorsal portion with 850-5C pBabe or 850-5C IL-8 cl19 (1×10^7 cells/mouse grown in adherence condition or thyrospheres derived cells $1x10^6$). Tumor diameters were measured at regular intervals with a caliper. Tumor volumes (V) were calculated with the formula: $V=A\times B_2/2$ (A=axial diameter; B=rotational diameter). This study was conducted in

accordance with Italian regulations for experimentation on animals. For xenograft histological analysis, Ki-67 antibody was from Biocare Medical, and CD31 antibody was from R&D Systems (USA).

3.10 Patients and specimens.

Series of patients was constituted by 30 cases of papillary thyroid carcinomas were obtained from the files of Pathology Unit, Istituto Nazionale Tumori of Naples. All cases of thyroid carcinomas and normal thyroid tissues were reviewed according to WHO classification criteria, using standard tissue sections and appropriate immunohistochemical slides. Medical records for all cases of thyroid carcinomas were reviewed for clinical information.. The following clinical and pathological parameters were evaluated for each tumor included in the study: patient age at diagnosis; tumor size; histologic subtype; nuclear grade; nodal status (number of positive lymph nodes); tumor stage; tumor recurrence or distant metastasis.

3.11 Tissue microarray building

A thyroid Multi-Tumor Array (TMA) was constructed using 30 tumor tissue samples, using the most representative areas from each single case. All tumors and controls were reviewed by an experienced pathologist (R.F.).
Two tissue cylinders from each case with a diameter of 0.6 mm were punched from morphologically representative tissue areas of each ‘donor’ tissue block and brought into one recipient paraffin block (3×2.5 cm) using a semiautomated tissue array (Galileo TMA).

3.12 Immunohistochemistry analysis

Immunohistochemical staining was performed on slides from formalin-fixed, paraffin embedded tissues, corresponding to cases thyroid carcinomas and normal thyroid tissues to evaluate the expression of Tryptse and OCT4 markers. Then, paraffin slides were deparaffinized in xylene and rehydrated through graded alcohols. Antigen retrieval was performed with slides heated in buffer EDTA (pH 6 for tryptase and ph8 for OCT4) in a bath for 20 minutes at 97°C. After antigen retrieval, the slides allow to cool. The slides were rinsed with TBS and the endogenous peroxidase was inactivated with 3% hydrogen peroxide. After protein block (BSA 5% in PBS 1x), the slides were incubated with primary antibody to anti-human Tryptase (Dako, Glostrup, Denmark 1:100) and Oct4 (Abcam Rabbit Anti-Human Oct4 clone EPR2054 1:500) for 1 hour. The sections were rinsed in TBS and incubated for 20 min with Novocastra Biotinylated Secondary Antibody (RE7103), a biotin-coniugated secondary antibody formulation that recognized goat and rabbit

immunoglobulins. Then the sections were rinsed in TBS and incubated for 20 min with Novocastra Streptavidin-HRP (RE7104) and then peroxidase reactivity was visualized using a 3,3'-diaminobenzidine (DAB). Finally, the sections were counterstained with hematoxylin and mounted. For each sample, at least five fields (inside the tumor and in the area exhibiting tumor invasion, 400 X400) and >500 cells were analyzed.

3.13 Statistical Analysis

The results are expressed as the mean ± SEM of at least 3 experiments. Values from groups were compared by using the paired Student *t* test or the Duncan test. *P* value < 0.05 was considered statistically significant. The association between Oct4 and Tryptase expression with other clinico-pathological parameters was conducted using the χ^2. The level of significance was defined as $P<0.05$. All the statistical analyses for IHC were carried out using Statistical Package for Social Science v. 20 software (SPSS Inc., Chicago, IL, USA).

4. RESULTS

4.1 MC conditioned medium induces EMT in thyroid cancer cell

Recently, we have shown that human thyroid carcinoma cells are a powerful chemoattractant for mast cells (MC) and this effect requires thyroid carcinoma (TC)-cell-derived VEGF-A. Moreover, human TC cells induce MC degranulation and cytokine synthesis *in vitro*. MC-released mediators enhanced proliferation, survival and invasive behavior of TC cells *in vitro*; the co-injection of MC and TC cells accelerated the growth of TC cell xenograft in athymic mice. Consistently, the inhibition of MC function by a specific inhibitor, sodium cromoglycate (Cromolyn), blocked tumor expansion by inhibiting proliferation of cancer cells. Finally, we found that human papillary thyroid carcinomas (PTCs) feature a remarkable MC infiltrate, whose density correlates with tumor invasinveness (Melillo et al. 2010); it is possible that the acquisition of invasive phenotype underlies the induction or enhancement of the EMT process. To investigate whether MC have a role in promoting-enhancing EMT, we used different cell lines: human SV40-immortalized thyroid cells Nthy-ori (or called Nty-ori 3.1), human anaplastic thyroid carcinoma cells (ATC) 850-5C; and TPC1, derived from a human papillary thyroid carcinomas (PTC); MC lines, LAD2 and HMC-1 derived, respectively, from a human mastocytosis and from a human MC leukemia expressing a constitutively active c-Kit mutant (Butterfield et al., 1988) and human primary MCs derived from human lung (HLMC).

We treated human thyroid normal and cancerous cell lines with HMC1-derived conditioned media (MC CM), non-conditioned medium (DMEM, Dulbecco's Modified Eagle Medium), or exogenous TGF-β, a known inductor of EMT, used as a positive control, for 24h *(Fig 9)* and evaluated cell morphology. Both normal and cancer thyroid cells show a classical epithelial morphology when cultured in their growth medium. By contrast, cells cultured in MC CM or in the presence of TGF-β showed a striking morphological change. These cells displayed loss of epithelial morphology, became spread, and acquired a mesenchymal phenotype, reminiscent of EMT. Since similar data were obtained by using LAD2 and HLMC conditioned medium (not shown), we decided to use HMC-1 CM (MC CM) for all the following experiments, and named HMC-1 cells MC throughout the thesis.

During EMT, cell–cell adhesion must be lost to produce individual migrating mesenchymal-like cells. This is mainly achieved through the suppression of E-cadherin. Various transcription factors such as SNAIL (SNAIL1), SLUG

(SNAIL2), SIP-1, ZEB-1, E12/E47 (Peinado et al., 2004), and Twist1/2 (Yang et al., 2004), bind the promoter of the *E-cadherin* gene to repress its transcription. The EMT transcription factors have been shown to repress other tight junction proteins such as claudins, occludin, and ZO-1 (Ikenouchi et al., 2003; Ohkubo and Ozawa, 2004). The acquisition of mesenchimal features is also determined by the expression of mesenchimal markers such as N-cadherin, fibronectin, vimentin and β-catenin. To verify whether MC CM can induce molecular markers of EMT in Nthy-ori, TPC-1 and 850-5C cell lines, these cells were treated with MC CM for 24h, after which they were harvested and the expression of EMT markers was evaluated by Quantitative Real Time Polymerase Chain Reaction (Q-PCR).

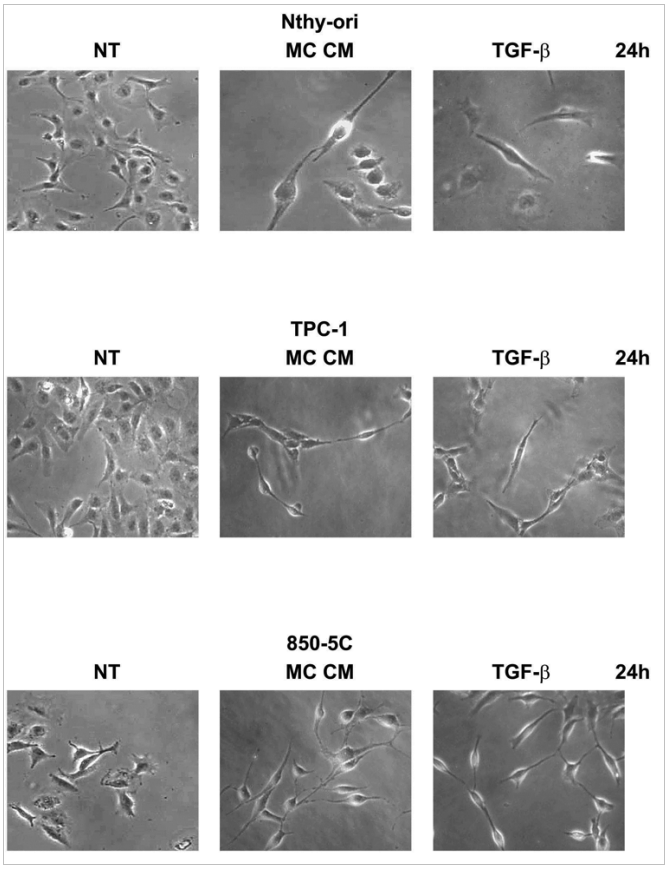


Fig. 9 Morphological appearance of Nthy-ori, TPC-1 and 850-5C cell lines after 24h treatment with non-conditioned culture medium (NT), HMC-1 Conditioned Medium (MC CM) or exogenous TGF- β (50ng/ml).

Some EMT markers were overexpressed in thyroid cancer with respect to Nthy-ory cell line in basal conditions and each cell line displayed a peculiar pattern of gene expression (not shown). However, as shown in *Fig.10*, the mRNA expression level of several EMT markers (Twist1, SLUG, SNAIL, ZEB-1 and Vimentin) increased after MC CM treatment while E-Cadherin, the

epithelial cell marker, decreased in treated with respect to untreated thyroid cells. A significant induction of mRNAs encoding SNAIL in TPC-1 cell line and Twist1 in 8505-C cell line was observed. Moreover, Nthy-ori cells display a strong increase of Twist1, SLUG and Vimentin. A decrease in E-Cadherin mRNA levels was observed in the three cell lines.

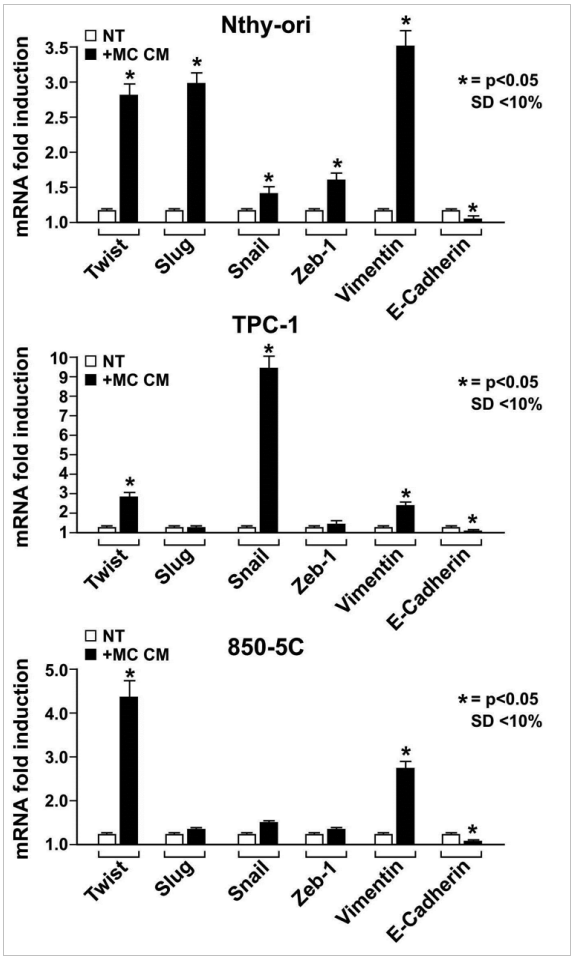


Fig. 10 Evaluation of EMT marker mRNA levels by Quantitative Real Time Polymerase Chain Reaction in Nthy-ori, TPC-1 and 850-5C cell lines after 24h treatment with MC CM, NT: untreated cells.

Western blot analysis (*Fig.11*) was used to confirm the induction of selected EMT proteins. To this aim, we performed a time-course experiment (1h-24h), and verified that the protein levels of the transcriptional regulators SLUG, SNAIL, and ZEB-1 accumulated with different kinetics in the three cell lines. In particular, SLUG increase preceded ZEB-1 and SNAIL induction in Nthy-ori cells. In TPC-1 and 850-5C cells, ZEB-1, SLUG and SNAIL progressively accumulated. The differences observed in the kinetics of induction of EMT transcription factors may reflect differences in the genetic background or/and in the malignancy of in the three cell lines. Whatever the case, the results

obtained indicate that MC CM is a strong inducer of EMT in thyroid immortal and malignant cells.

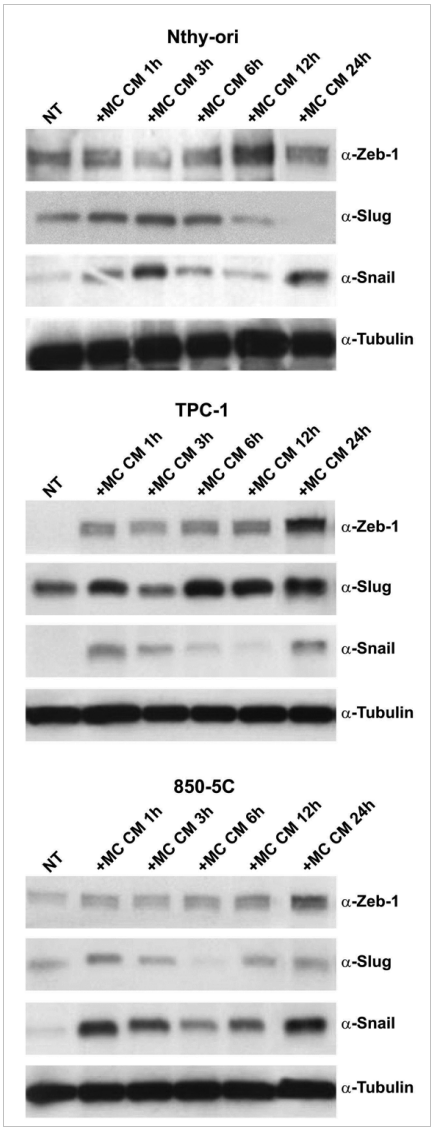


Fig. 11 Protein expression of the EMT transcription factors (SLUG, SNAIL, ZEB-1) analyzed by Western Blot analysis in Nthy-ori, 850-5C and TPC-1 cell lines. Anti-tubulin monoclonal antibody was used as a control for equal loading.

It is well documented that matrix metalloproteases (MMPs) are involved in EMT through three distinct mechanisms: (a) elevated levels of MMPs in the tumor microenvironment can directly induce EMT in epithelial cells, (b) cancer cells that undergo EMT can produce more MMPs, facilitating cell invasion and

metastasis, and (c) EMT can generate activated stromal-like cells that drive cancer progression via further MMP production (Evette S Radisky 2010). Matrix metalloproteinases (MMPs) degrade and modify the extracellular matrix (ECM) as well as cell-ECM and cell-cell contacts, facilitating detachment of epithelial cells from the surrounding tissue. They differ from each other in their structural domain architecture, substrate specificity, temporal and tissue specific expression patterns. MMPs were also named for their preferred substrates within the extracellular matrix (ECM): collagen-cleaving MMPs (MMP-1, -8, and -13) were designated collagenases, gelatin (denatured collagen)-cleaving MMPs (MMP-2 and -9) were termed gelatinases, and MMPs that degraded a broad spectrum of ECM proteins were called stromelysins (MMP-3, -10, and -12) or matrilysins (MMP-7). MMPs play key functions in embryonic development, but they are also up-regulated in cancer, where they stimulate tumorigenesis, cancer cell invasion and metastasis. In order to verify whether MC CM was able to induce MMP expression in our system, we performed a Q-PCR for various MMPs, in basal conditions and upon treatment with MC CM for 24h, 48h and 72h. As shown in *Fig.12*, we found that MC CM treatment caused the upregulation of selected MMPs in thyroid cell lines. In fact, an increased expression of MMP12 in Nthy-ori cell line after 72h treatment with MC CM was observed. Moreover, TPC-1 cells displayed a strong increase of the mRNA level of MMP3, that peacked at 48h treatment with MC CM, while in 850-5C cells MMP10 was the main up-regulated MMP.

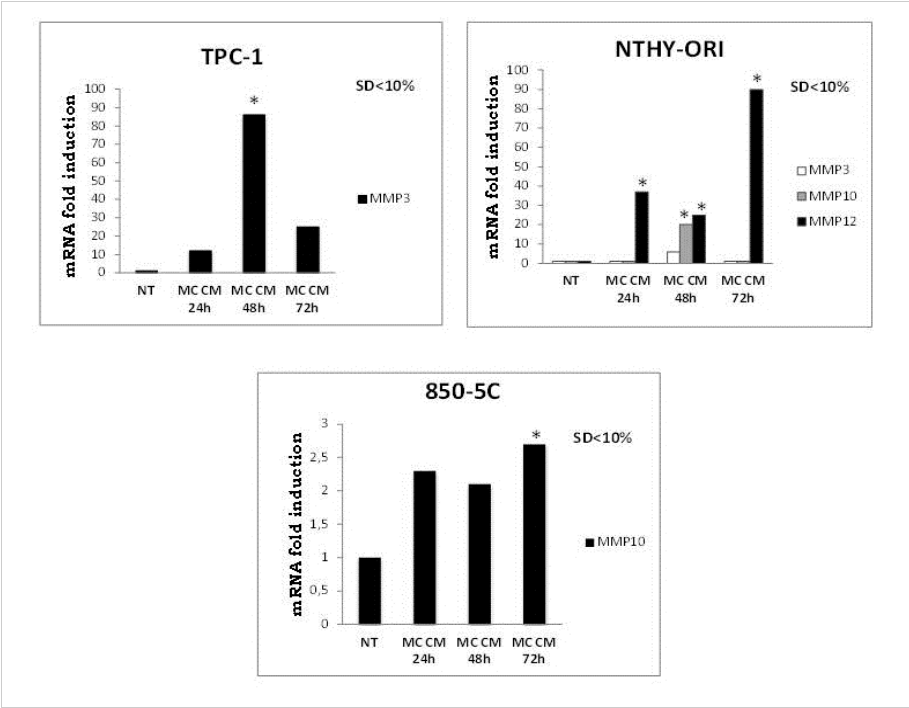


Fig. 12 Quantitative Real Time Polymerase Chain Reaction of Nthy-ori, TPC-1 and 850-5C cell lines treated or not with MC CM for 24h, 48h and 72h to evaluate the mRNA levels of MMPs ($p<0.05$).

EMT is also characterized by the acquisition of reduced intercellular adhesion and increased motility of epithelial cells. These features can be evaluated through matrigel migration and wound healing assays. The invasive potential of cancer cells is usually assessed *in vitro* using Matrigel as a surrogate basement membrane and Wound healing assay, that mimics cell migration during wound healing in vivo. By using Matrigel invasion assays, we showed that the invasive ability of cancer cell lines treated with MC CM dramatically increased compared with cells cultured in regular medium (not shown). Moreover, we used wound healing assays. The basic steps of these assays involve creating a "wound" in a cell monolayer, capturing the images at the beginning and at regular intervals during cell migration to close the wound, and counting the cell migrated over the wound to quantify the migration rate of the cells. With these experiments, we showed that the ability of thyroid cells to close wounds in a cell monolayer was significantly increased by MC CM treatment, as shown in *Fig.13*.

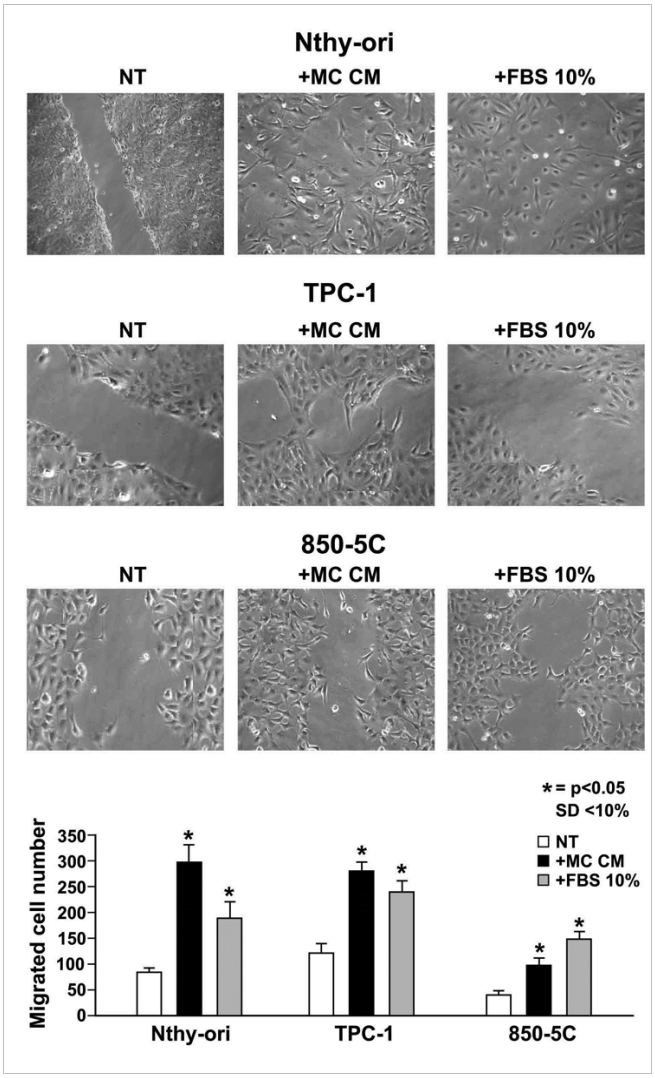


Fig.13 Wound Healing assay on Nthy-ori, 850-5C and TPC1 cell lines treated or not with MC MC for 12h and growth medium additioned with 10% FBS as a positive control. Representative photomicrographs of the wounded cell monolayers are shown. The number of migrated cells over the wound in each condition was calculated. ($p<0.05$)

MC CM was as effective as complete medium (DMEM 10% FCS) in wound closure, indicating that MC CM contains soluble factors that can induce functional EMT.

4.2 MC CM treatment causes the loss of tight junctions in TC cell lines.

Loss of epithelial markers and disruption of the cell-cell junction are hallmarks of EMT. The tight junction (TJ) is an important structure that determines epithelial cell polarity and disappears during EMT. TJs constitute the epithelial junctional complex, together with adherens junctions (AJs) and desmosomes,

located at the most apical part of the complex. TJs create the primary barrier to the diffusion of solutes through the paracellular pathway and maintain cell polarity as a boundary between the apical and basolateral plasma membrane domains (Schneeberger and Lynch, 1992). The molecular architecture of TJs consist in three closely related proteins, ZO-1, ZO-2 and ZO-3, that form the undercoat structure of TJs together with other peripheral membrane proteins. The constituens of strand themselves are the integral membrane proteins Claudins and Occludin; Claudins are 20- to 27-kD integral membrane proteins with 4 hydrophobic domains, 2 extracellular loops and N- and C-terminal cytoplasmic domains. The extracellular loops are critical for homophilic and/or heterophilic interactions with the other TJs proteins and the formation of ion-selective channels. The intracellular C-terminal domain is involved in anchoring Claudin to the cytoskeleton through interactions ZO-1, ZO-2 and ZO-3. Currently, 24 claudin genes encoding for the respective proteins have been identified in human subjects; among them, Claudin-1 is one of the major structural and functional elements of TJs as it plays a role in paracellular transport, cellular growth, differentiation and is critical for the manteinance of cellular polarity. Recent studies show that Claudin-1 is aberrantly expressed in diverse types of human cancer and has a causal role in EMT in human cancers (Junichi Ikenouchi et al. 2003).

To examine whether MC CM affects the expression of epithelial cell markers and the integrity of on TJs in thyroid cells, we first performed a FACS analysis for Claudin-1 and Cytokeratin in Nthy-ori and TPC-1 cell lines. As shown in *Fig.14 A*, MC CM treatment reduced Claudin-1 an Cytokeratin expression in TPC-1 and Nthy-ori cell lines. This phenomenon was less evident in 850-5C cells due to their constitutive low levels of both proteins (not shown).

To confirm this finding and to evaluate the structure of TJs, we performed an immunofluorescence staining of TPC-1 cell line treated or not with MC CM with specific anti-Claudin-1 antibodies. As shown in *fig.14 B*, untreated TPC-1 cells exhibited a regular linear labeling of Claudin-1 at sites of cell-cell contact regions, indicating that TJs are maintained in this cell line. However, MC CM caused a dramatic decrease of the protein and a disruption of TJs.

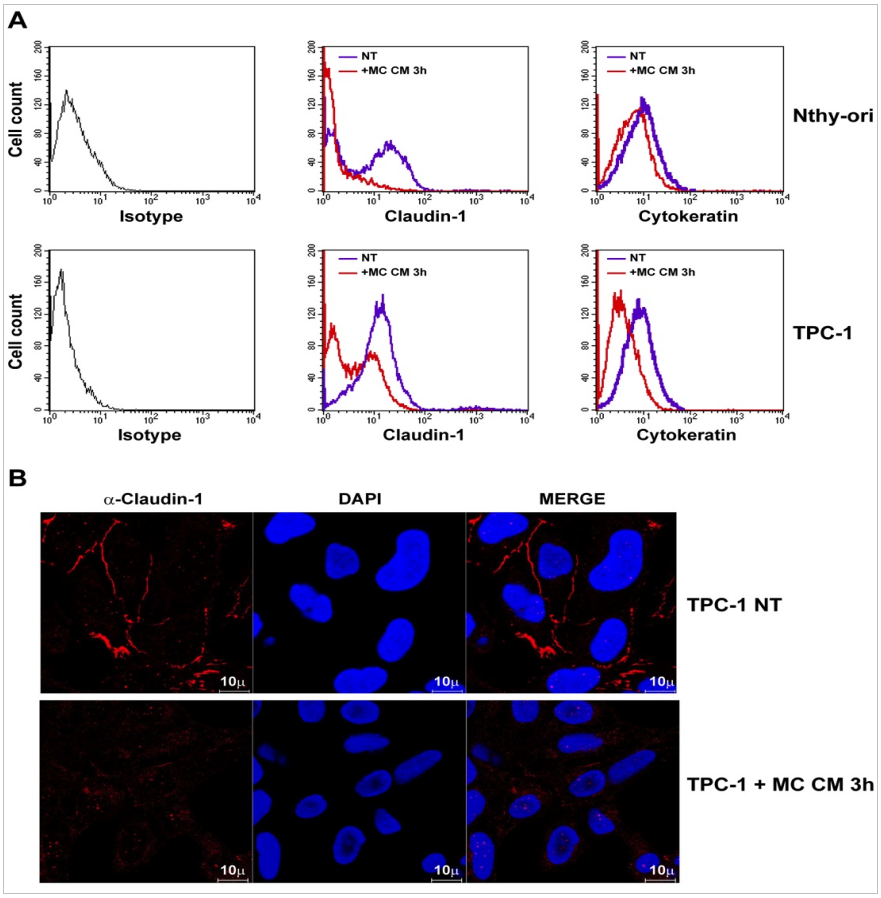


Fig. 14 A) FACS Analysis on Nthy-ori and TPC-1 cells treated or not with MC CM for 3h by using anti.Claudin-1 and anti-pan-Cytokeratin antibody. B) Immunofluorescence microscopy of TPC-1 cells with antibody specific for Claudin-1. In MC CM treated cells Claudin-1 became undetectable in the cell-cell contact regions. Bars, 10 μm.

4.3 MC CM treatment of human thyroid cancer cells causes the activation of AKT and STAT3 pathways

To determine which signaling pathway was involved in MC-mediated EMT induction, we next treated thyroid cells with MC CM and evaluated the activation of various signaling pathways, including MAPK/ERK, JNK, NFkB, PI3K/AKT and STAT3 by western blot analysis with antibodies that specifically recognize activated isoforms of signalling components of the indicated pathways *(Fig. 15)*. Among the various pathways tested, we found that AKT and STAT3 were significantly activated upon MC CM stimulation in each cell line. In particular, 850-5C cell line displayed a remarkable activation of AKT that peaked at 1h of MC CM treatment and then decreased. On the other hand, phospho-STAT3 protein expression levels increased at 1h of MC CM treatment and accumulated up to 12 hours. In TPC-1 cell line, we found

that phospho-STAT3 and phospho-AKT protein expression levels increased after 1h of MC CM treatment and persisted for several hours. Finally, in Nthy-ori we observed the activation of STAT3 that persisted for many hours, while MEK activation was transient. In both TPC-1 and 850-5C cell lines we did not appreciate increase in phospho-MEK levels, probably due to the very high basal levels displayed by these cells.

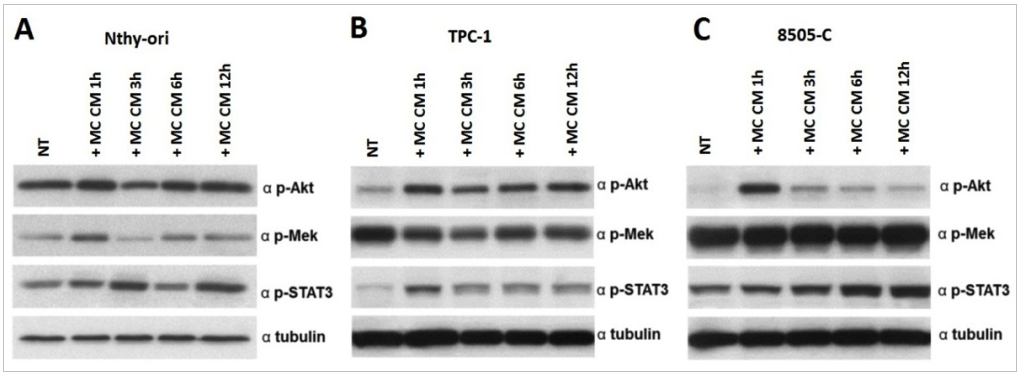


Fig. 15 Western Blot analysis of Nthy-ori (A), TPC-1 (B) and 850-5C (C) cell lines treated or not with MC CM for 1h, 3h, 6h and 12h with anti-phospho-AKT, anti-phospho-MEK and anti-phospho-STAT3 antibodies. Anti-tubulin monoclonal antibody was used as a control for equal loading.

To assess whether AKT and/or STAT3 activation is necessary for EMT induction/enhancement, we evaluated, by wound healing assay, MC CM-induced EMT in TPC-1 in the presence or in the absence of U0126, LY-294002, or S31-20, inhibitors of MEK, AKT and STAT3 activity, respectively (*Fig.16A*). Sodium Chromoglycate (Cromolyn), an inhibitor of MC degranulation, was used as a control. As shown in *Fig.16 B,* the inhibition of each pathway negatively affected wound closure induced by MC CM. We obtained the same results also in Nthy-ori and 850-5C cell lines (not shown).

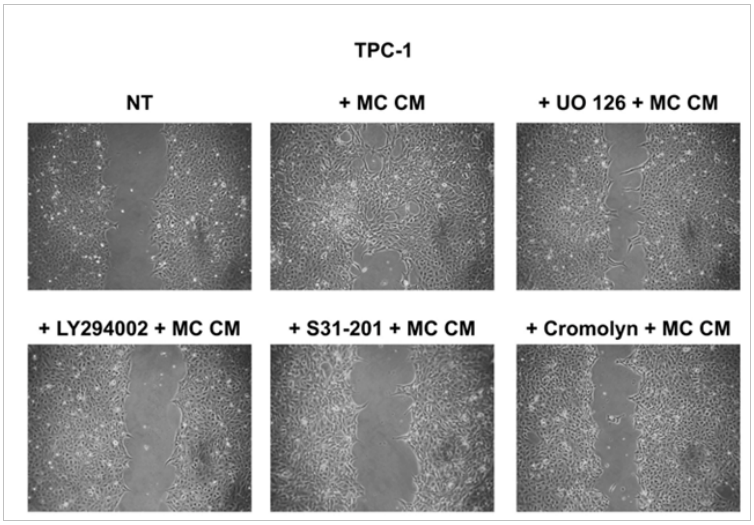


Fig. 16 Wound healing assay performed in TPC-1 cell line treated, for 1h, or not with only MC CM and with inhibitors S31-201 (100 μM), LY-294002 (50μM), U0126 (20 μM) and Cromolyn (10 μM), as a control.

4.4 MC-derived factors (IL-6, IL-8 and TNF-α) can induce of EMT in human thyroid cell lines.

MC can produce many different soluble mediators, both in resting conditions and upon activation. We have previously shown that TC cells can stimulate MC to degranulate and produce soluble factors with protumorigenic biological activity (Melillo et al., 2010). Thus, to mimik cancer microenvironment and to identify MC mediators of EMT, we exposed MC (HMC-1, LAD2 and HLMC) to TPC-1 and 850-5C cell CM (TC CM). We defined such cells as "tumor educated" MCs (TEMC). TEMC were evaluated for cytokine production through RT-PCR and ELISA cytokine arrays. By merging data from the two different approaches, we consistently identified many cytokines and chemokines produced by MCs whose levels could be induced by TC CM, including OPN, IL-1β, IL-6, IL-8, TNF-α, CXCL1, and CXCL10 (not shown). However TGF-β, a know inductor of EMT, was not produced by TEMC. We also verified whether MC CM caused an increase in TGF-β levels in TC cells, by performing an ELISA assay. The data indicated that TGF-β levels did not change in MC CM-treated with respect to untreated TC cells (data not shown), indicating that MC CM-induced EMT is not mediated by an induction of TGF-β in TC cells.

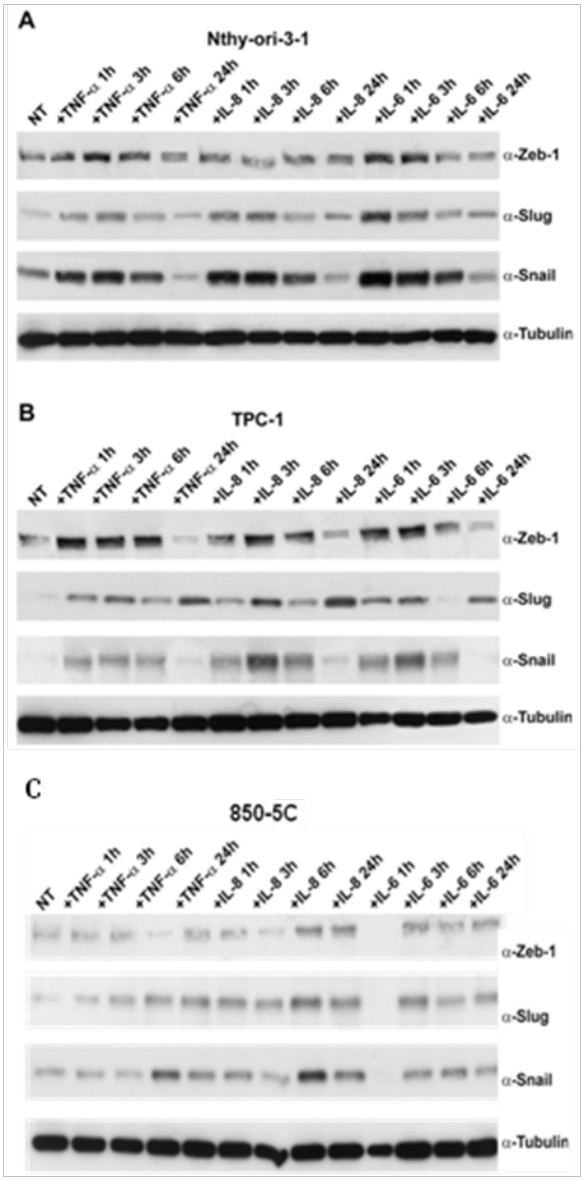


Fig.17 Western Blot analysis of Nthy-ori (A), TPC-1 (B) and 850-5C (C) cell lines treated or not with TNF-α, IL-8 and IL-6 (100ng/ml) at different time points to evaluate ZEB-1, SLUG and SNAIL proteins expression levels. Anti-tubulin monoclonal antibody was used as a control for equal loading.

Based upon on these data, we supposed that some of the identified cytokines might be the mediators of EMT in our system; for this reason, we tested the capability of each cytokine to induce EMT in our cells. The expression of biochemical markers of EMT (SNAIL, SLUG, ZEB-1) was evaluated by Q-PCR (not shown) and western blot analysis on Nthy-ori, TPC-1 and 850-5C cell lines *(FIG.17)*. We found that, among all the cytokine tested, only TNF-α, IL-6 and IL-8 could induce EMT. The other mediators that we tested,

including OPN, CXCL12, IL1β, MIF-1, CXCL1 and CXCL10 were either less powerful or uncapable of inducing EMT transcriptional factors. To further investigate the functional role of IL-6, IL-8 and TNF-α in EMT, we performed a wound healing assay. Nthy-ori and TPC-1 (*Fig. 18)* cell lines were treated with each cytokine or with MC CM, as a positive control. As shown in this figure, IL-8 induced wound closure similarly to MC CM. IL-6 and TNF-α were less efficient than IL-8 in inducing wound healing. We obtained the same results in 850-5C cell line (not shown).

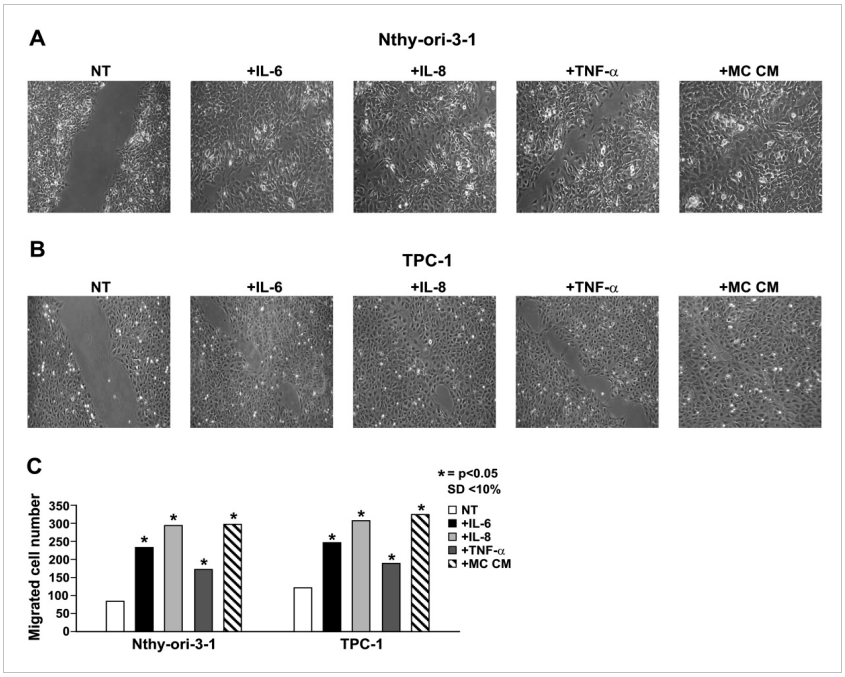


Fig.18 Wound healing assay on Nthy-ori (A) and TPC-1 (B) cell lines treated with IL-6, IL-8, TNF-α (50 ng/ml) or with MC CM as a positive control. C) The number of migrated cells over the wound in each condition was calculated ($p<0.05$).

To confirm the requirement for these factors in MC CM-induced EMT, each cytokine was inactivated through immunodepletion with specific antibodies. Each cytokine was recognized by the specific antibody which, in turn, was bound to Protein G-Sepharose beads. The immune complexes were then removed from MC CM by centrifugation. Immunodepletion deprived MC CM form each cytokine, without significantly affecting the levels of the others, as assessed by ELISA assays (not shown). A representative experiment performed by using immunodepleted MC CM on Nthy-ori cells is shown in *Fig. 19*. After cells reached confluence, we created wounds in the confluent plates; cells were then treated with non-conditioned culture medium (NT), complete MC CM or MC CM immunodepleted of IL-8, IL-6 and TNF-α. We found that the blockade of IL-8 by neutralizing antibodies inhibited the ability of MC CM to induce wound repair.

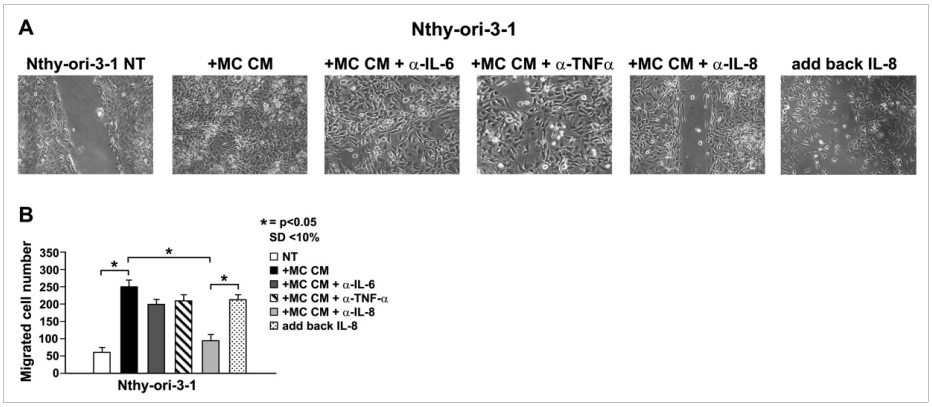


Fig. 19 A) Wound healing assay with immunodepleted MC CM on Nthy-ori cell line. The removal of each factor from MC CM reduced wound closure of Nthy-ori cells. However, IL-8 depletion was more effective than IL-6 or TNF-α removal. B) The number of migrated cells over the wound in each condition was calculated.

IL6- and TNFα-immunodepletion showed less significant effects. To further underline the importance of IL-8, we observed that IL-8 add-back to IL-8-immunodepleted MC CM restored cell migration. These findings indicate that IL-8 is necessary to induce EMT in thyroid cells.

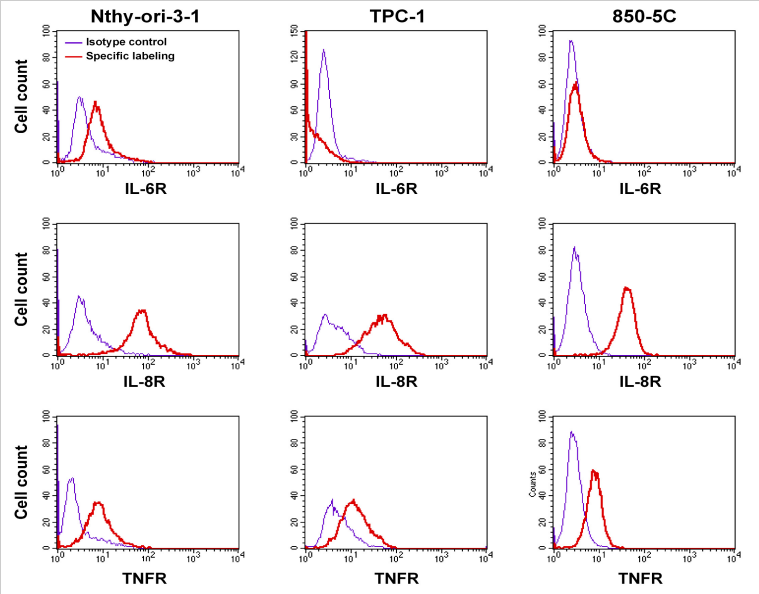


Fig.20 Fluorescence Activated Cell Sorting (FACS) analysis to estimate cell-surface expression of IL-6R, CXCR1 and TNFR in Nthy-ori, TPC-1 and 850-5C cell lines.

Despite IL6 and TNF-α were also present in MC CM and capable of inducing EMT in Nthy-ori, their ability to induce wound healing was lower than that of IL-8. Interestingly, despite TNF-α receptors (TNFR) are expressed on Nthy-ori, TPC-1 and 850-5C cell lines, IL-6R is expressed only in Nthy-ori, as shown in *Fig.20*. However, all the cell lines expressed high levels of the IL-8 receptors CXCR1 *(Fig.20*) and CXCR2 (data not shown).

4.5 IL-8 stimulation of TC cells causes an expansion of stem cell population.

The relationship between EMT and CSCs has been well documented in different types of cancer; EMT inducers or regulators could also induce cancer cells to acquire stem cell-like characteristics, indicating that there is a crosstalk between the EMT program and the pathways involved in regulating stemness (Mani *et al*. 2008). Since IL-8 is the most relevant inducer of EMT in our system, we asked if IL-8 has any role in the acquisition of stemness features or in the expansion of stem cell population in TC cells. One of the recognized features of CSC is their higher aldehyde-dehydrogenase activity with respect to the other cells. It has been demonstrated that cells derived from TC tissues contain a small population of high aldehyde-dehydrogenase (ALDH) activity. TC cells with high ALDH expression ($ALDH^{high}$) possessed the ability to self-renew and re-initiate serial transplantable tumors that recapitulated the

phenotype and metastatic behavior of the parental tumors (Todaro et al. 2010). In another study, (Klonisch et al. 2009) ALDH^{high} cell subpopulation with potential stem-like characteristics in the ATC cell line 850-5C was identified.
Thus, we first assessed the percentage of TC cells with high aldehyde-dehydrogenase (ALDH) activity by using the Aldefluor assay, that uses a fluorescent non-toxic substrate for ALDH, which freely diffuses into intact and viable cells, and under the enzyme activity is converted into a metabolite, which is retained inside the cells. The amount of fluorescent reaction product is proportional to the ALDH activity in the cells and thus to the number of stem cells. Screening of Nthy-ori, TPC1 and 850-5C cells revealed, in each cell line, a distinct population with high aldefluor activity (ALDH ^{high} cells) that was more abundant in the 850-5C, derived from a human ATC; TPC-1 and Nthy-ori cell lines displayed a low level of ALDH ^{high} cells (*Fig.21*).

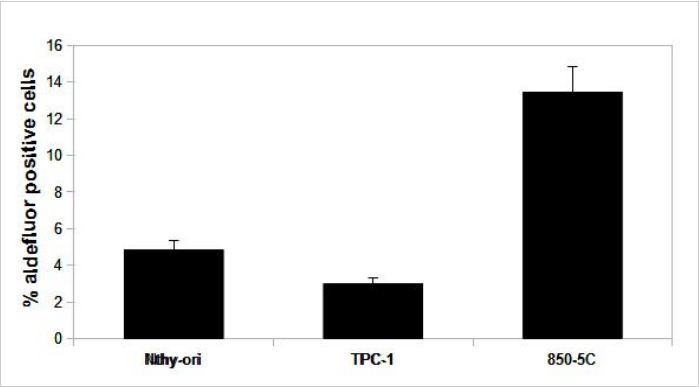
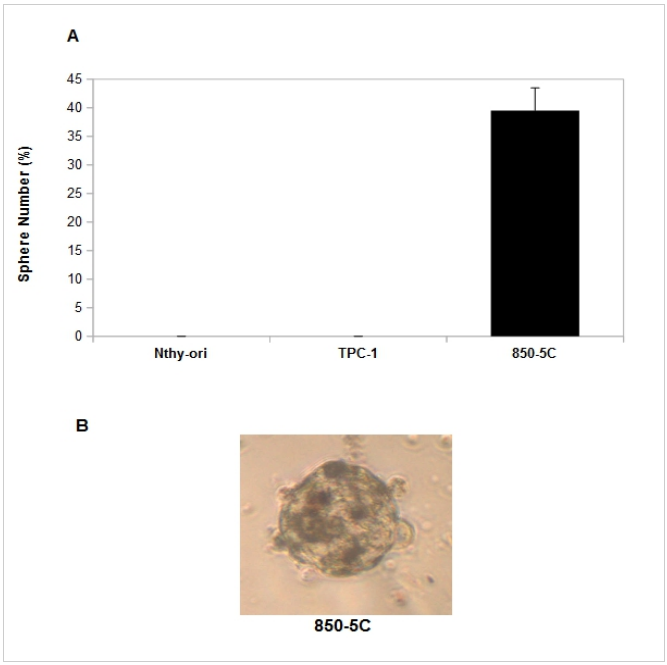


Fig. 21 Percentage of ALDH^{high} cells in freshly isolated serum deprived thyroid cells (Nthy-ori, TPC-1 and 850-5C); cells were permeabilized with the Cytofix/Cytoperm Kit (BD). Identification of ALDH^{high} cells was performed using the ALDEFLUOR kit (StemCell Technologies). As a negative control, an aliquot of cells from each sample was treated with 50 mmol/L of diethylaminobenzaldehyde, a specific ALDH inhibitor. Intracellular fluorescent product was measured by flow cytometry and sorting gates.

Another way to isolate cells with stem-like features is to exploit their ability to growth in low adherence conditions, which favours the formation of cell spheroids. Sphere-forming assays have been widely used to identify CSCs. This assay is based on the evaluation of floating non-adherent multicellular aggregates obtained in suspension culture or cultures under non-adhesive conditions. Cells with stem-like properties are capable to form spheroids under anchorage-independent conditions in a special serum-free medium enriched for EGF (*epidermal growth factor*) and bFGF (*basic fibroblast growth factor*) growth factors. In fact, a small number of cells grew out as floating spherical colonies, which were termed “thyrospheres”, after 7-10 d of culture. During the next few days, thyrospheres progressively augmented their volume because of increased cell number. We found that 850-5C, but not TPC-1 or Nthy-ori cells

formed spheres with high efficiency *(Fig. 22)*, accordingly with previous observations (Shimamura et al. 2014).

Fig.22 A) Sphere-forming assay. Quantification of sphere number by the formula (number of formed spheres/number of wells containing cells) x100 in Nthy-ori, TPC-1 and 850-5C. B) Morphological appearance of a thyrosphere from 850-5C cells.



To confirm that thyrospheres were indeed enriched in stem cells, we compared the levels of stemness markers in 850-5C cells grown in adherence or in low-adherence conditions. 850-5C thyrospheres displayed higher mRNA levels of nestin, ABCG2 and ALDH, assessed by QPCR, in comparison to adherent cells *(Fig. 23)*.

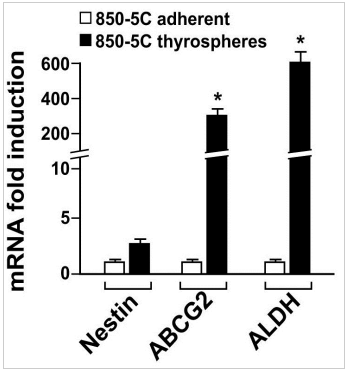


Fig. 23 Real Time PCR for stem cell markers (Nestin, ABCG2 and ALDH) in 850-5C thyrospheres in comparison with 850-5C grown in adherence condition ($p<0.05$).

To evaluate whether IL-8 receptors were expressed in 850-5C spheroids, we performed FACS analysis with anti-CXCR1 and -CXCR2 antibodies in adherent and sphere-forming cells. This analysis showed that 850-5C thyrospheres were enriched in CXCR1 and CXCR2 with respect to adherent cells *(Fig. 24)*.

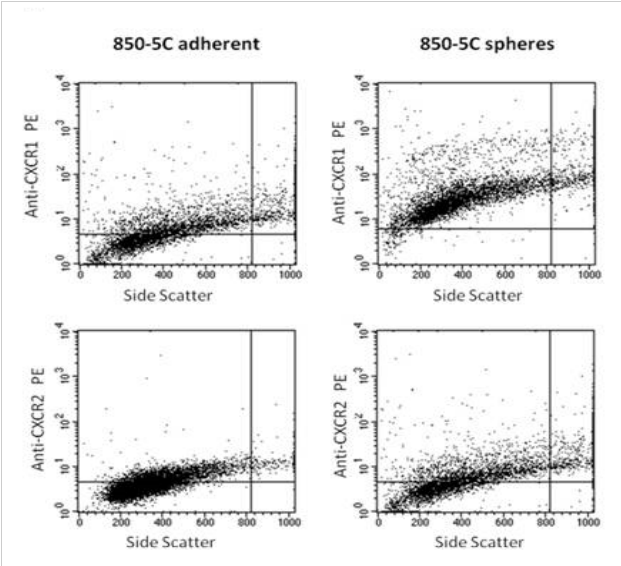


Fig. 24) FACS analysis of adherent 850-5C and 850-5C-derived thyrospheres to evaluate CXCR1 and CXCR2 expression.

To assess whether IL-8 could induce/potentiate TC cel stemness, cells were stimulated with IL-8 and stem cell phenotype was evaluated. IL-8 treatment significantly increased the percentage of ALDH^{high} cells with respect to untreated Nthy-ori, TPC-1 and 850-5C cells. Moreover, IL-8 stimulation caused an increase of stem cell markers (OCT4, SOX2, ABCG2 and NANOG) with respect to untreated thyroid cells; TGF-β was used as a positive control *(Fig.25 A-B)*.

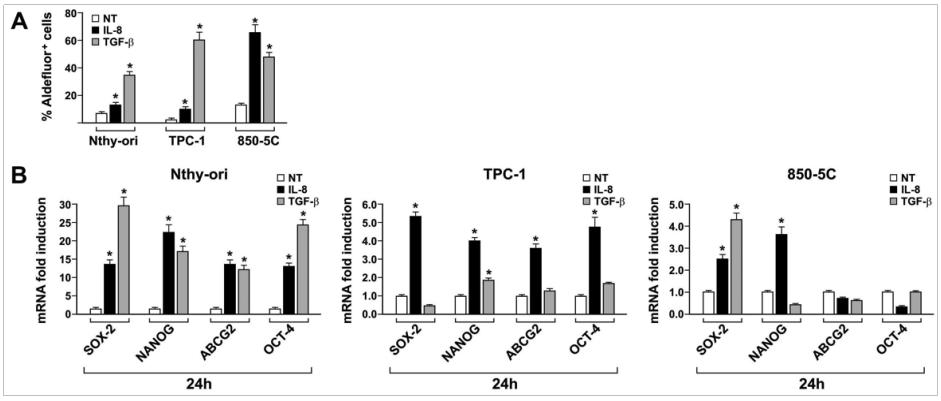


Fig. 25 A) Percentage of $ALDH^{high}$ cells. Thyroid cells (Nthy-ori, TPC-1 and 850-5C untreated, treated with IL-8 50ng/ml or TGF-β 50ng/ml for 24h were permeabilized with the Cytofix/Cytoperm Kit (BD). Identification of $ALDH^{high}$ cells was performed using the ALDH kit (StemCell Technologies). As a negative control, an aliquot of cells from each sample was treated with 50 mmol/L of diethylaminobenzaldehyde, a specific ALDH inhibitor. Intracellular fluorescent product was measured by flow cytometry and sorting gates. B) Q PCR to evaluate the expression levels of stem cell markers in thyroid cell lines treated or not with TGF-β (50ng/ml), or IL-8 (50ng/ml) for 24h.

As shown in *Fig.26,* IL-8 stimulation induced sphere formation with higher efficiency (number and dimension, *Fig 26 B-C)* in 850-5C compared to unstimulated cells. These spheres were bright, smooth-edged, and compact and were clearly different from irregular clumps of cells. Figure *26-A* shows representative photograms reporting the average size of spheres after 15 days of culture in low-adherent conditions.

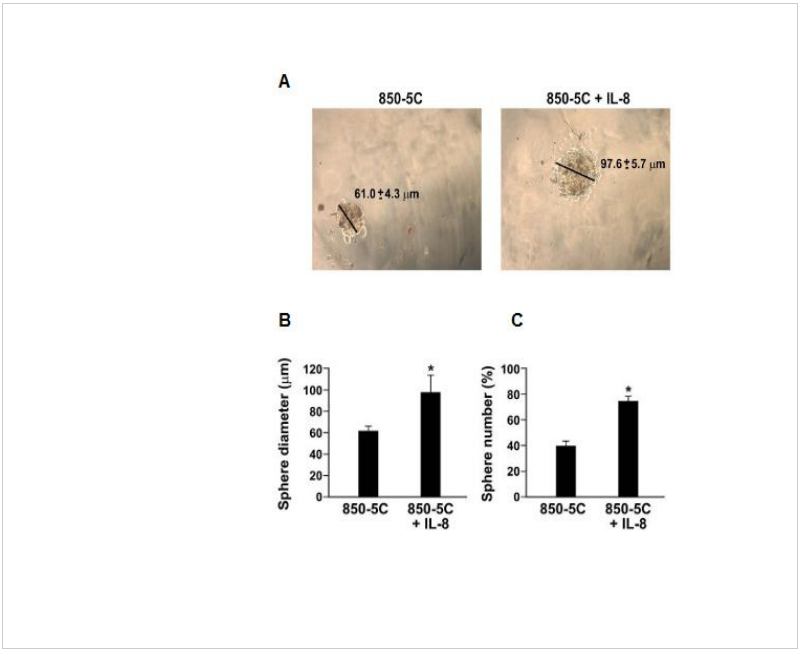


Fig.26 Sphere-forming assay. A) Morphological appearance and average diameters of thyrospheres from 850- 5C cells treated or not with IL-8 (50 ng/ml). B) Quantification of sphere size (mean diameter in μm) from 850-5C cells treated or not with IL-8 (50 ng/ml). C) Quantification of sphere number by the formula (number of formed spheres/number of wells containing cells) x100 ($p<0.05$).

By analysing a panel of TC cell lines, we found that they all express, to a different extent, detectable levels of IL-8 and of its receptors CXCR1/2, suggesting that endogenous IL-8 may be important for intrinsic stemness features of these cells. Accordingly, the ability of each cell line to form spheres, a typical stemness feature, correlated with endogenous IL-8 levels (data not shown). In *Fig. 27*, a representative ELISA assay to evaluate IL-8 levels in the cell lines used in this study is shown.

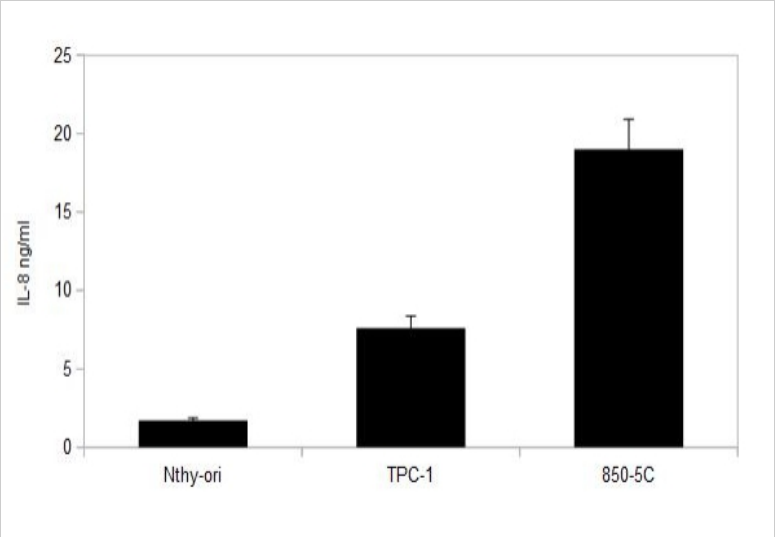


Fig. 27 Elisa assay to evaluate endogenous IL-8 content in Nthy-ori, TPC-1 and 850-5C cell line

Interestingly, we were also able to shown that IL-8 levels were higher in 850-5C thyrospheres with respect to adherent cells (*Fig. 28*).

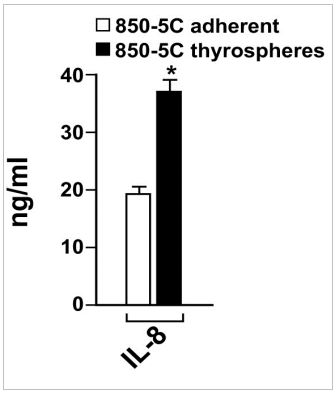


Fig. 28 ELISA assay to quantify the IL-8 content in 850-5C thyrosphere with respect to adherent cells.

Since we have shown that TC cells express endogenous IL-8 and its receptors CXCR1 and CXCR2, and that these proteins are enriched in thyrospheres, we aimed at demonstrating that stemness could be inhibited by targeting the IL-8/CXCR1/CXCR2 axis. At this end, we blocked this ligand-receptor circuit with neutralizing antibodies anti-IL-8 (2.5 μg/ml), anti-CXCR1(2.5 μg/ml) or anti-CXCR2 (1 μg/ml), and observed a dramatical reduction in sphere-forming ability of 850-5C cells, as evaluated in terms of sphere number and size. As a negative control, we used isotype-matched antibody (anti-HA) (*Fig.29*). These results suggest that an autocrine IL-8/CXCR1/CXCR2 axis is necessary for thyroid CSCs manteinance.

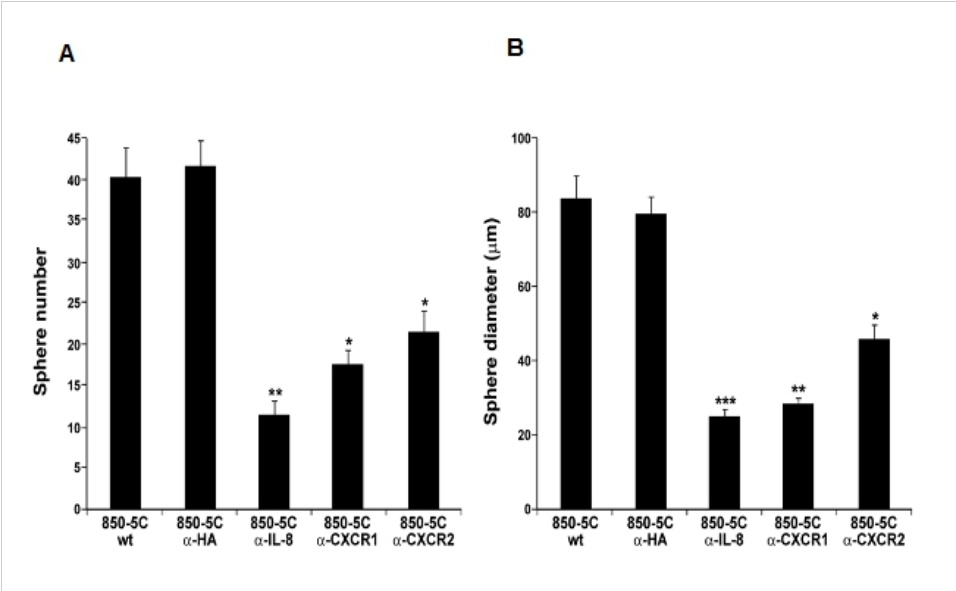
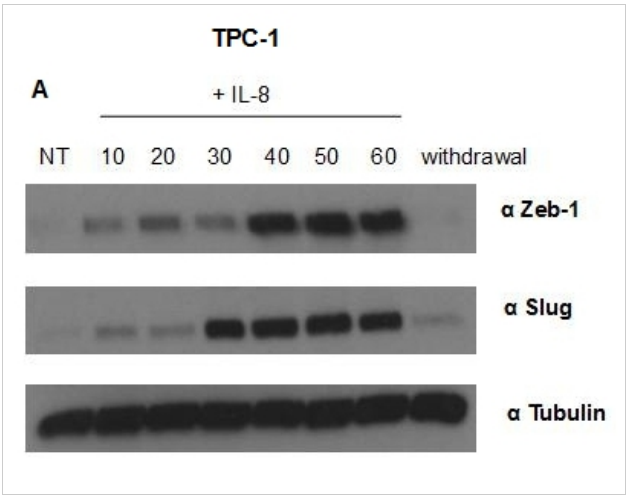


Fig.29 The inhibition of IL-8/CXCR1-CXCR2 axis impairs sphere forming ability of thyroid cancer cells. Anti-IL-8, anti-CXCR1, anti-CXCR2, anti-HA: isotype-matched antibody as negative control. Number (A) and diameter (B) of formed spheres are decreased when 850-5C cells are treated with anti-IL-8 or anti-CXCR1 and anti CXCR2. Sphere number (%) was calculated by the formula (number of formed spheres/number of wells containing cells) x100. (*= $p<0.05$; **=$p<0.01$, ***=$p<0.001$)

5.6 850-5C engineered to ectopically express IL-8 undergo EMT and display stemness features.

In order to obtain a stable EMT phenotype in thyroid cancer cells, we stimulated TPC-1 cell line, that displayed low basal levels of EMT markers, with TNFα, IL-6 or IL-8 up to 60 days; we found that IL-8, but not the other two cytokines (not shown), induced a sustained expression of the EMT markers SLUG and ZEB-1, but not SNAIL. Moreover, we evaluated, by QPCR, the expression levels of stemness markers, and showed that again, IL-8, but not TNFα or IL-6, could induce sustained expression of various stemness genes. However, IL-8 withdrawal dramatically decreased the levels of EMT and stemness markers *(Fig. 30A-B)*. These data indicate that the continuous stimulation with IL-8 is required to induce a stable EMT phenotype and stemness features in TC cells.



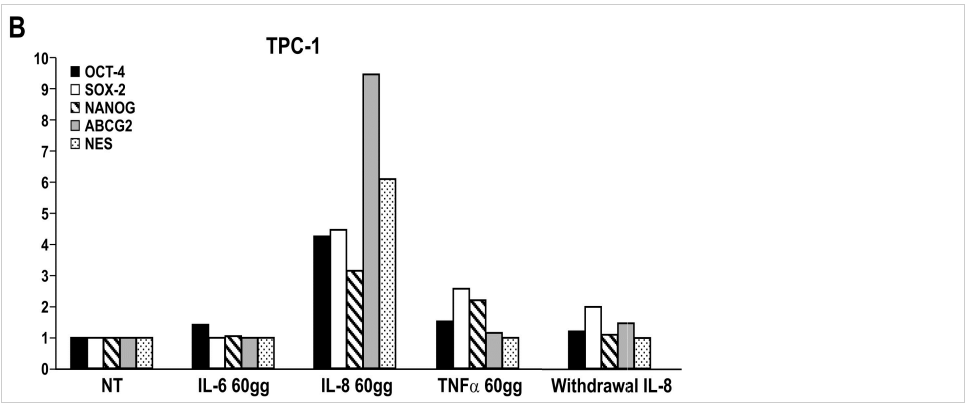


Fig.30 A) Western blot analysis for ZEB-1, SLUG, SNAIL in TPC-1 untreated (NT), treated with IL-8 (50 ng/ml) and upon IL-8 withdrawal (withdrawal IL-8). B) Real Time PCR for stem cell markers performed in the same conditions as in A).

In order to obtain cells chronically exposed to IL-8, we generated 850-5C engineered to ectopically express the cytokine (850-5C IL-8); as a control, we used empty vector transfected cells (850-5C pBabe). IL-8 transfected cells were subjected to antibiotic selection and isolated clones were then screened for IL-8 expression by ELISA assay *(Fig.31A)*. Based upon these results, we selected 850-5C IL-8 clone 19 and clone 23 for further investigations.

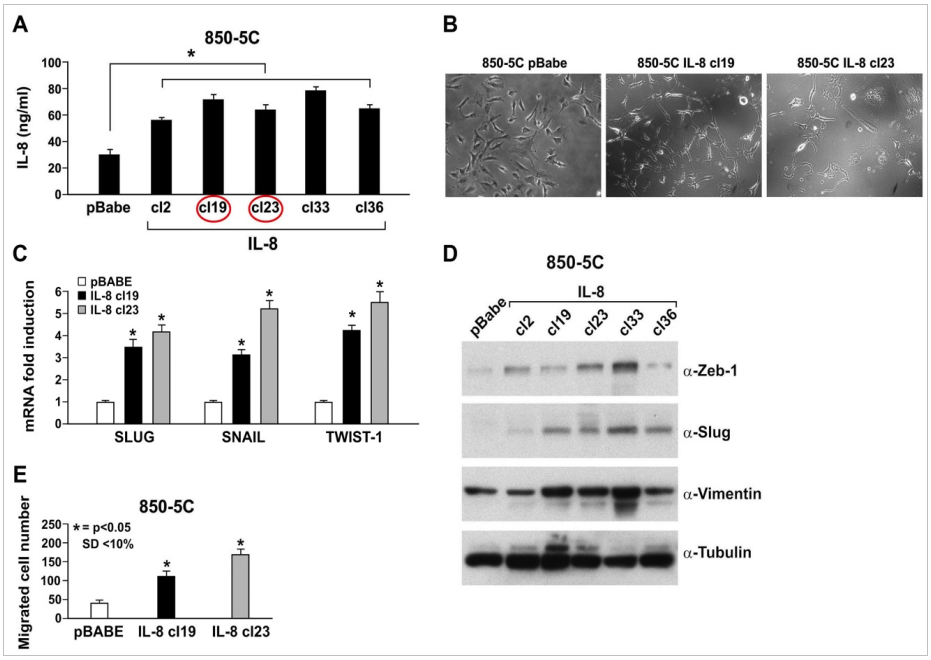


Fig.31 A) Elisa assay to evaluate the IL-8 content in conditioned media of 850-5C overexpressing IL-8 compared with empty vector-transfected cells (pBabe). B) Morphological appearence of 850-5C overexpressing IL-8 and relative pBabe clones. C) Real Time PCR for EMT transcription factors in 850-5C IL-8 clones with respect to control clones (pBabe). D) Western blot analysis for EMT markers (ZEB-1, SLUG, SNAIL and Vimentin) in the same clones; anti-tubulin is used for equal loading. E) Wound Healing assay in 850-IL-8 clones with respect to pBabe clones: the number of migrated cells over the wound was calculated.

850-5C IL-8 cells showed a fibroblast-like morphology, typical of EMT *(Fig. 31 B)* and displayed overexpression of EMT markers at protein and mRNA levels with respect to control clones *(Fig. 31 C-D);* finally, we investigated the ability of IL-8-transduced cells to migrate in wound healing assays. As shown, in *Fig. 31 E,* 850-5C IL-8 clones were able to close the wound more efficiently than 850-5C pBabe cells. We then studied stemness features of 850-5C IL-8 clones by Aldefluor assay; the percentage of $ALDH^{high}$ cells was more abundant in the two 850-5C IL-8 clones than in control cells, and 850-5C IL-8 clones displayed enriched expression of stem cell markers, compared to control cells, as tested by Q-PCR analysis (*Fig. 32 A, B*).

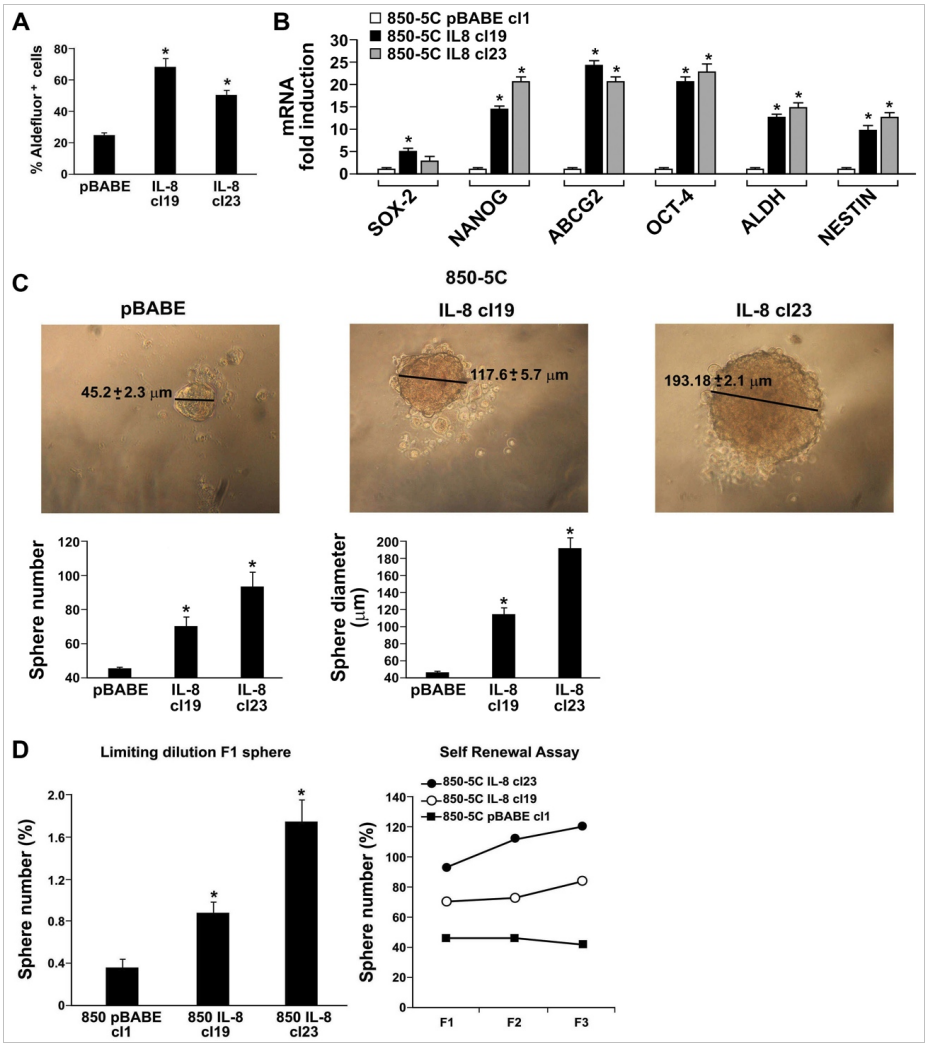
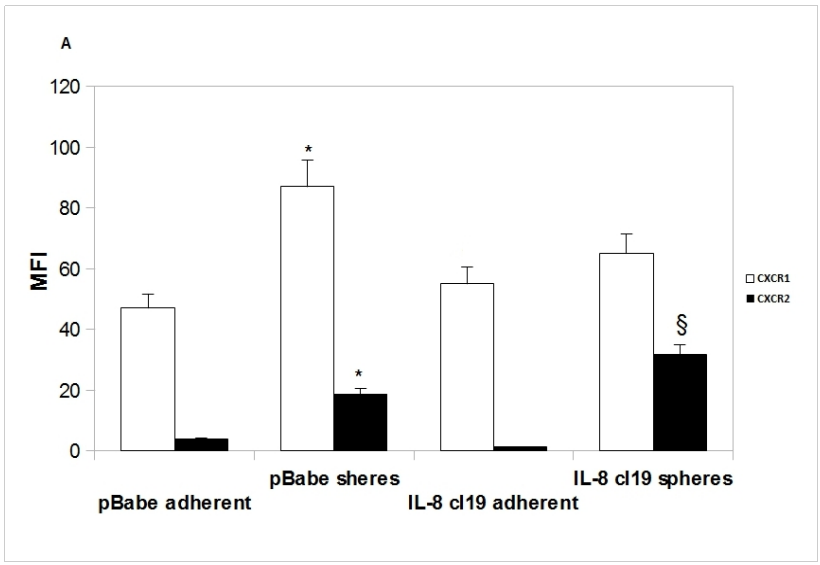


Fig. 32 A) Cytofluorimetric analysis of ALDH^{high} cells. 850-5C IL-8 clone 19 and clone 23 displayed higher ALDH activity than control cells. B) Q-PCR showed elevated levels of stem cell markers in 850-5C IL-8 cl19 and cl23 with respect to control cells. C) Sphere-forming assay: morphological appearance and average diameter of thyrospheres from 850-5C IL-8 clone 19 and clone 23. Quantification of sphere number was obtained by the formula (number of formed spheres/number of wells containing cells) x100. Quantification of sphere size (mean diameter in μm). D) Sphere-forming assay under limiting dilution conditions. 850-5C IL-8 clones showed a higher sphere forming frequency than control cells. Quantification of sphere number by the formula (number of formed spheres/number of wells containing cells) x100. Self-renewal assay. 850-5C IL-8 clones show an increase in sphere number (%) in subsequent generations; by contrast 850-5C sphere-forming ability decreases at F3. Sphere number (%) was calculated by the formula (number of formed spheres/number of wells containing cells) x100. ($p<0.05$)

In *Fig.32 C,* we showed that sphere-forming ability of 850-5C IL-8 clones was higher than that of 850-5C pBabe cells, in terms of average number and diameter, providing further evidences of increased stemness features induced by IL-8 overexpression. To determine the number of sphere-initiating cell contained in a single thyrosphere, we performed a sphere-forming assay under limiting dilution condition. When single F1 spheres reached the diameter of 70-

80 μm, they were disaggregated in single cells and plated at the density of a single cell/well on ultralow-adhesion 96-well plates, using limiting dilution, verified microscopically. Frequency of formed thyrospheres were estimated after ~15 days. We found that 850-5C IL-8 clone 19 and clone 23 displayed a frequency of 0.9-1.1% and 1.8-2.0% sphere-initiating cells, respectively, whereas 850-5C pBabe showed a frequency less than 0.4%. In normal tissues, stem cell number is constant, because they divide asymmetrically. By contrast CSCs possess the ability to expand in number, as occurs during development or, in the adult, after tissue injuries, a property that cannot be accounted by asymmetric divisions. Recent findings in invertebrates demonstrated that increased numbers of SCs can be achieved through rounds of ‘‘symmetric self-renewing divisions,’’ whereby each SC produces two new cells with identical SC fate and proliferation potential (Morrison and Kimble, 2006). Loss-of-function mutations of cell polarity and cell fate determinants induce CSCs to divide symmetrically, leading to SC number increase, tissue overgrowth, and, ultimately, tumors (Cicalese et al. 2009). To determine the self-renewal capacity of sphere-forming cells we performed a self renewal assay *(Fig.32 D-Self renewal assay)*. 850-5C IL-8 and 850-5C pBabe cells were plated in stem cell culture medium in 96-well plates at the density of 15 cells per well. This primary generation of spheres was indicated as First generation (F1). The spheres were serially passed through 3 generations, to assess their self-renewal capacity *in vitro*. When F1 thyrospheres from 850-5C IL-8 and 850-5C pBabe grew to 70-80 μm in diameter, spheres were collected and disaggregated, and then re-plated in the same conditions (15 cells/well) in order to form new spheres and to obtain F2 and, subsequently, F3 thyrospheres. We counted the formed spheres for three generations and we found that 850-5C IL-8 clones displayed an increased sphere number formation in F2 and F3 generations, whereas 850-5C pBabe decreased thyrosphere-forming ability at F3. As shown in *Fig.33* 850-5C IL-8 and pBabe cell-derived thyrospheres displayed a similar enrichment in CXCR1-CXCR2 levels (*Fig.33 A)* with respect to adherent cells, as assessed by FACS analisys. However, IL-8 is involved in maintaining stemness, as 850-5C IL-8 thyrospheres exhibit higher expression of stemness markers with respect to those derived from pBabe cells *(Fig. 33B)*.



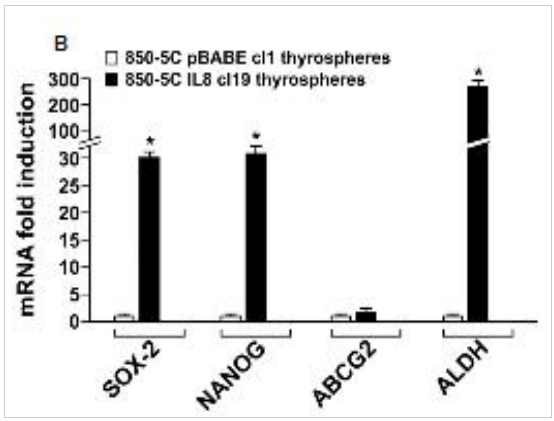
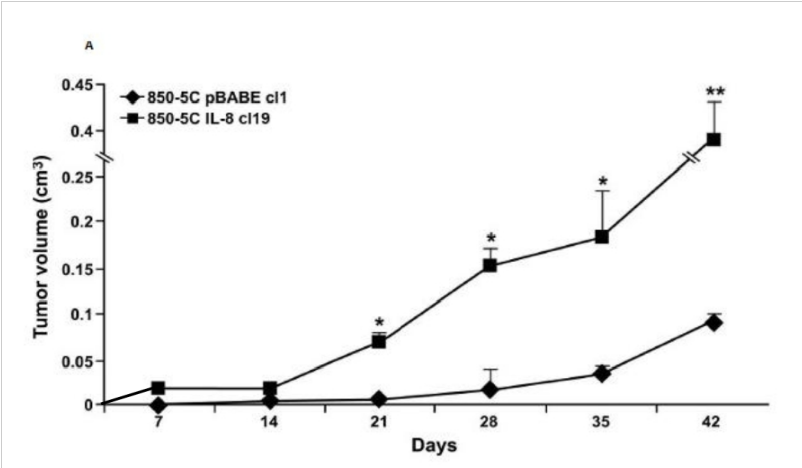


Fig. 33 A) Mean Fluorescence Intensity (MFI) of CXCR1 and CXCR2 performed by FACS analysis in 850-5C pBabe and 850-5C IL-8 cl 19 grown in adherence condition with respect thyrospheres (*=$p<0.05$ CXCR1 and CXCR2 in thyrospheres with respect to pBabe adherent stained for CXCR1 and CXCR2 respectively; §=$p<0.05$ CXCR2 with respect to IL-8 cl19 adherent stained for CXCR2). B) We performed Q-PCR for stemness markers in 850-5C pBabe thyrospheres and 850-5C IL-8 cl 19 thyrospheres ($p<0.05$).

4.7 IL-8 increases the tumorigenic potential of 850-5C cells.

CSCs are believed to be responsible for tumor onset and metastasis. They can indeed inititiate tumors very efficiently, while the bulk cancer mass, containing remnant cells, is not able to do so. As we demonstrated that 850-5C IL-8 cells contain a higher percentage of CSCs with respect to 850-5C cells, and that 850-5C IL-8 stem-like cells are endowed with a more efficient ability to proliferate and self-renew, we decided to verify whether 850-5C IL-8 exhibited a higher tumorigenic ability in comparison with control cells. To assess tumor growth of 850-5C pBabe and 850-5C IL-8 cl19, we used xenograft injection into athimic mice. $1x10^7$ cells from each adherent cell population were injected sub-cutaneously into the right dorsal portion of 4-week-old female BALB/c nu/nu *mice* (The Jackson Laboratory). Mice were inspected for tumor growth on a weekly basis. Six weeks later, mice were killed and tumors were excised. 850-5C IL-8 injected mice showed a significant increase in tumor growth rate compared to control tumors *(Fig. 34 A);* no statistically significant differences were observed in tumor incidence (9/10 850-5C pBabe vs 10/10 850-5C IL-8 cl19).
To assess the reasons of the increased growth rate of 850-5C IL-8 with respect to control xenografts, excised tumors were subjected to haematoxilin-eosin (H&E) staining and immunohistochemistry (IHC) with Ki-67, CD31 and cleaved caspase3 antibodies. As shown in *Fig. 34*, the number of Ki-67+ cells was higher in 850-5C IL-8 than 850-5C pBabe xenografts, indicating that 850-5C IL-8 proliferated faster than control cells in xenografts, although this difference was not statistically significant *(p value>0.05)*. Moreover, vessel density, evaluated with anti-CD31 antibodies, was significantly higher in 850-5C IL-8 tumors with respect to 850-5C pBabe xenografts (*p value*<0.001). No statistically significant differences were observed in the percentage of cleaved-caspase 3 stained cells between the two xenograft types (*Fig. 34 C)*.



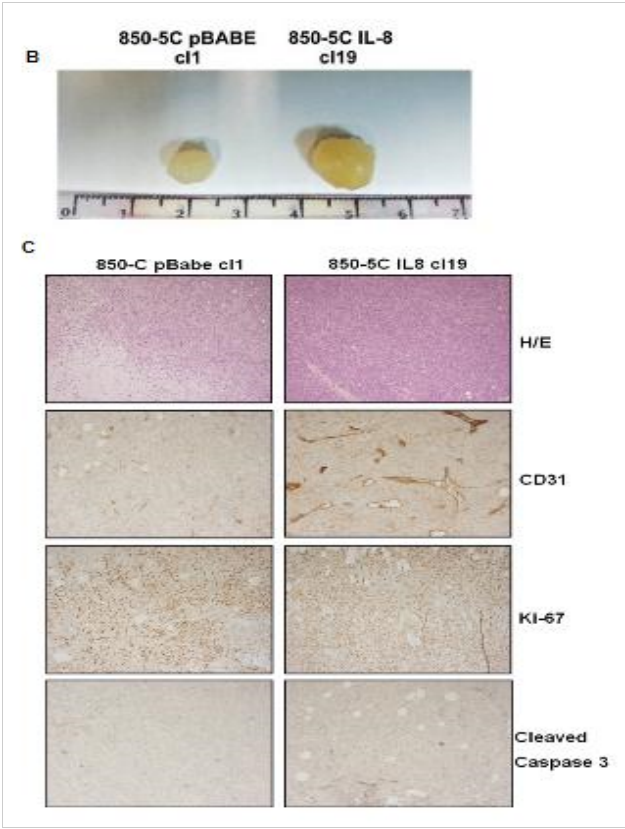


Fig. 34 A) Tumor volume of 850-5C pBabe and 850-5C IL-8 xenografts weekly measured (*p<0.05; **p<0.01). B) A representative picture of tumors excised at the end of the experiment is shown.
C) Representative 850-5C IL-8 and 850-5C pBabe xenograft sections (magnification 20x), Hematoxylin & Eosin analysis, staining by using different antibodies: Ki-67, CD31, Cleaved Caspase 3.

We next compared the tumorigenic potential of 850-5C pBabe and 850-5C IL-8 derived thyrospheres: 850-5C pBabe or 850-5C IL-8 cl19 thyrospheres were mechanically disaggregated into sigle cell suspensions and injected ($1x10^6$) into athimic mice. Tumor incidence (percentage of mice developing measurable masses), latency (time to develop measurable masses), and growth rate were assessed for 7 weeks *(Fig. 35)*.

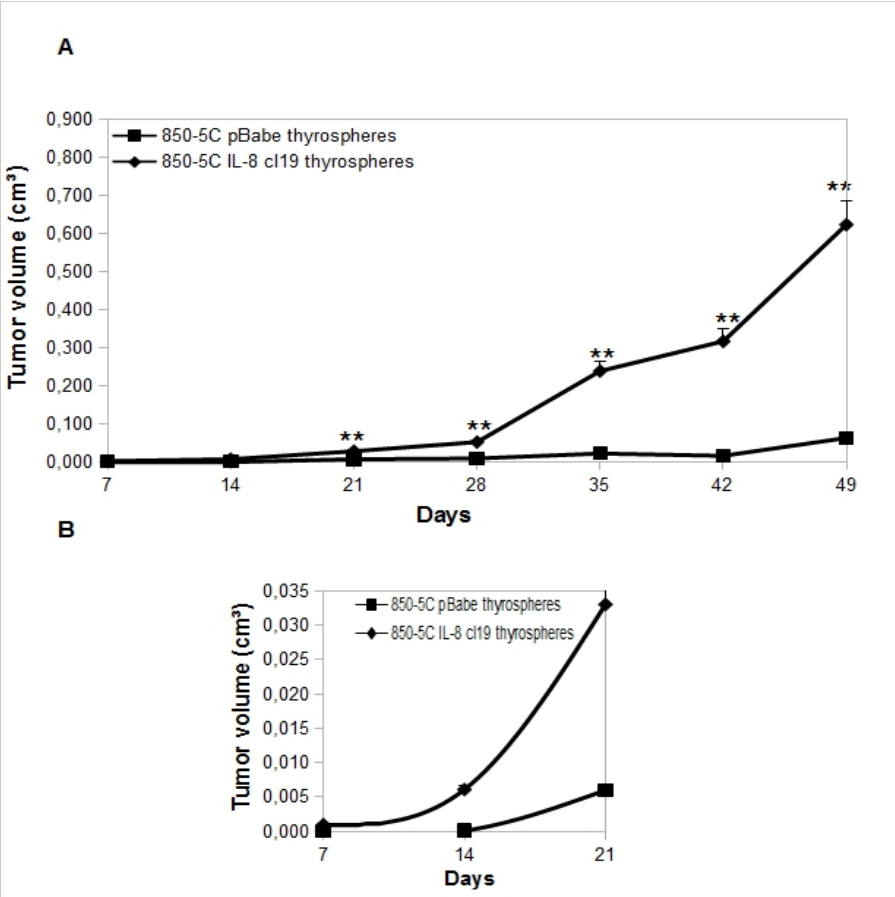


Fig. 35 A) Tumor volume of 850-5C pBabe and 850-5C IL-8 thyrosphere-derived xenografts weekly measured (**p<0.01). B) This graph showed the xenografts latency-mean time of thyrospheres derived-pBabe and IL-8 cl19.

850-5C IL-8 cl19 thyrospheres were more efficient in tumor initiation as 10/10 IL-8 cl 19 thyrosphere-injected mice formed tumors while only 6/10 850-5C pBabe thyrosphere-injected mice did (p=0,04 according to one-sided Fisher's exact test). Moreover, 850-5C IL-8 cl19 thyrosphere-derived xenografts revealed a significantly higher growth rate with respect to 850-5C pBabe thyrosphere-derived tumors ($p<0.01$) as final volume was 0,062 cm^3 for 850-5C pBabe and 0,623 cm^3 for IL-8 thyrosphere. 850-5C IL-8 cl19 thyrosphere-derived xenografts also exhibited a markedly decreased latency-mean time. In fact, 850-5C IL-8 cl19 thyrosphere-induced tumors appeared between 1 week (0,002 cm^3) and 14 days (0,006 cm^3) while 850-5C pBabe tumors only appeared at 14-21 days from injection ($p<0.05$) *(Fig.35 B)*.

4.9 MC density positively correlates with the stemness marker OCT4 in human papillary thyroid carcinoma specimens.

As we have shown that MC are a source of IL-8, and IL-8 is an inductor of EMT and stemness, we asked whether human thyroid carcinomas featuring a high density of MC also exhibited higher features of EMT and/or stemness. To this aim, a panel of 30 PTC specimens and 9 normal thyroids were collected, and a tissue microarray (TMA) was set up and used for IHC. Anti-Tryptase antibodies, which specifically identify MC, and anti-OCT4 antibodies, that detect a recognized marker of stem cells, were used *(Fig. 36)*. The age of patients ranged from 13–61 years, with an average age of 37 years. Tumors smaller than 2 cm (pT1) were 26,7% (8/30), tumors between 2 and 4 cm (pT2) were 6,7% (2/10), tumors larger than 4 cm (pT3) or with thyroid capsule invasion were 66,7% (20/30); lymph-node positivity was found in 56,7% (17/30) of patients at surgery. High OCT4 positivity was significantly associated with T (*p*-value= 0.010) (*Table 2*).

Parameters	OCT4		
T	**Low**	**High**	**P value**
T1 T2 T3	7(100%) 0 15(78,9%)	0 2(100%) 4(21,1%)	0,010
LNM			
Negative Positive	10(90,9%) 14(73,7%)	1(9,1%) 5(26,3%)	0,256
Capsule Invasion			
Negative Positive	8(80%) 16(80%)	2(20%) 4(20%)	1
Stage			
T1-T2 N0 Tx N1	3(100%) 21(77,77%)	0 6(22,22%)	0,361

Table 2. This Table summarizes the correlation between OCT4 expression and clinicopathological parameters in TMA of human PTC specimens analysed by immunohistochemistry.

Tryptase expression, was low in 12/30 cases and high in 18/30. Statistical analysis showed that Tryptase expression was significantly associated with T (*p* value=0,057) (*Table 3*).

PARAMETERS	TRYPTASE		
T	**Low**	**High**	**P value**
T1 T2 T3	5(71,4%) 0 5(26,3%)	2(28,6%) 2(100%) 14(73,6%)	0,057
LNM			
Negative Positive	4(36,4%) 8(42,1%)	7(63,4%) 11(57,9%)	0,76
Capsule Invasion			
Negative Positive	6(60%) 6(30%)	4(40%) 14(70%)	0,11
Stage			

T1-T2No TxN1	2(66,66%) 10(37,1%)	1(33,33%) 17(62,9%)	0,320

Table 3. This Table summarizes the correlation between Tryptase expression and clinicopathological parameters in TMA of human PTC specimens analysed by immunohistochemistry.

Moreover, we found a positive correlation between OCT4 and Tryptase expression *(p value= 0.025) (Table 4*).

	OCT4		
TRYPTASE	**Low**	**High**	**P value**
Low	12(100%)	0	
High	12(66,66%)	6(33,33)	0.025

Table 4. In this table we show the correlation between OCT4 and tryptase expression in human PTCs.

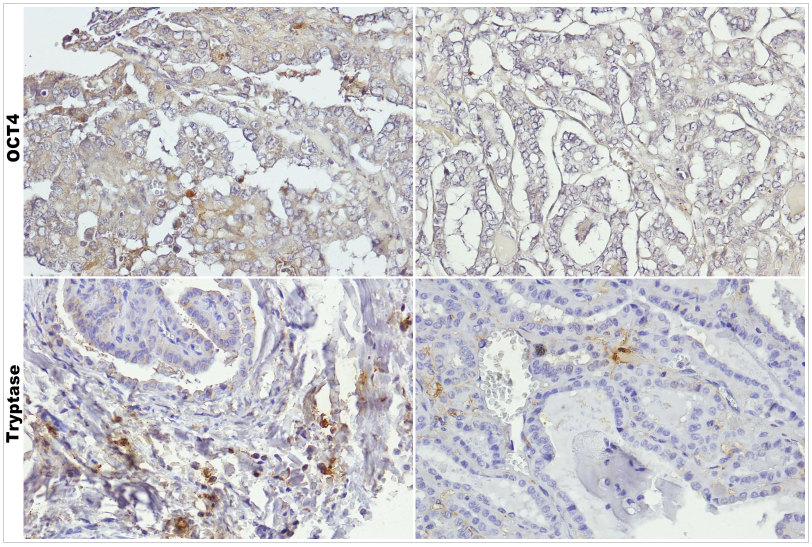


Fig. 36 Immunohistochemistry of human papillary thyroid cancer specimens with anti-OCT4 antibody and Tryptase staining (brown).

4.8 SLUG can induce EMT and stemness in PTC (TPC-1) thyroid cancer cells.

As SLUG expression was maintained by long-term IL-8 treatment, and 850-5C IL8 clones constitutively expressed high SLUG levels, we supposed that it might be an important mediator of IL-8 biological activity and sufficient to induce EMT and stem-like features in TC cells. We selected the TPC-1 cell line, as they featured very low SLUG expression and ALDH activity, to generate cells with ectopical expression of this transcription factor. We obtained a mass population (mp) and isolated various clones with high SLUG expression (*Fig. 37*).

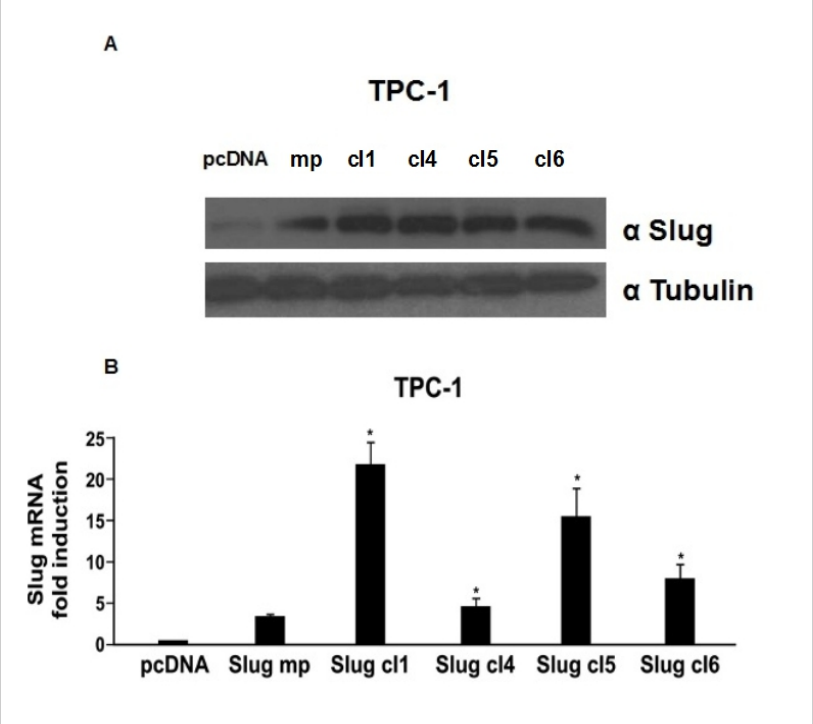


Fig. 37 A) SLUG protein levels in TPC-1 clones as assessed by western blot. B) SLUG mRNA expression in TPC-1 SLUG clones by Q-PCR

SLUG expression in TPC-1 cells enhanced their EMT features. In fact, TPC-1 SLUG cells (cl5) displayed a mesenchimal-like morphology (*Fig. 38 A)*, and were more efficient in wound closure *(Fig.38 B)*

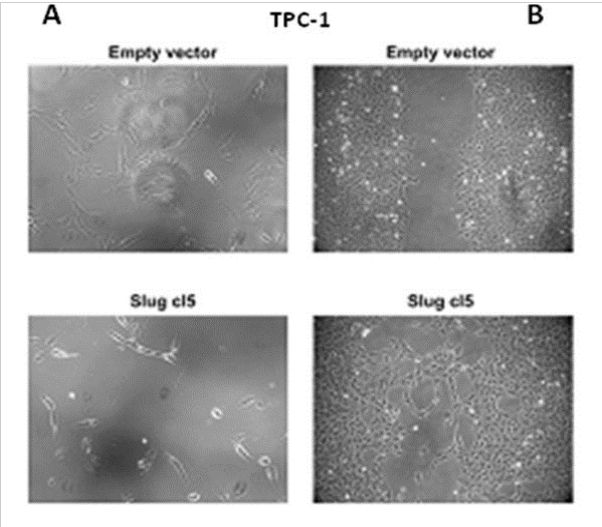


Fig.38 A) Morphological appearance of TPC-1 SLUG cl5 with respect to empty vector cells; B) Wound Healing assay shows that SLUG- expressing TPC-1 cells are more efficient in wound closure than controls.

Moreover, TPC-1 SLUG cells featured an higher percentage of aldefluor positive cells, upregulation of stemness markers, as shown by Q-PCR assay, and higher sphere-forming ability with respect to control clones (*Fig. 39)*.

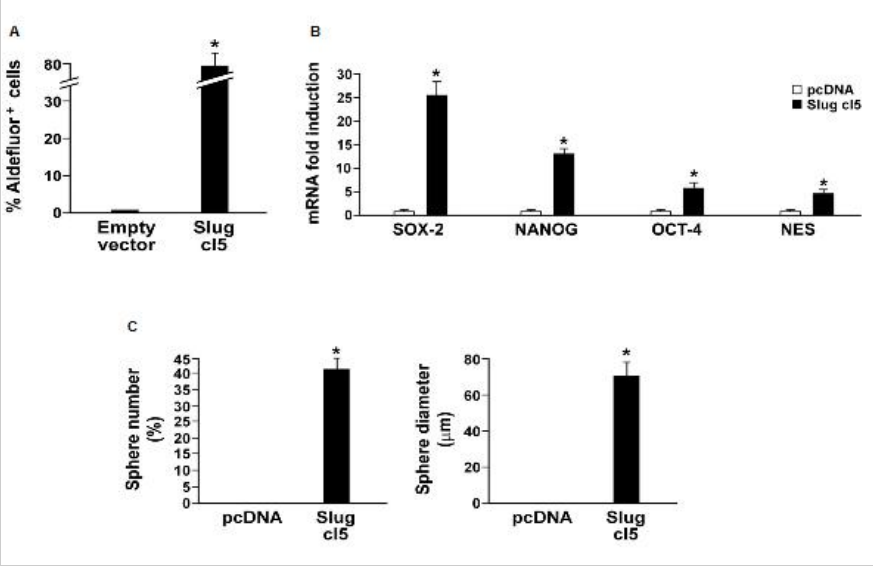


Fig. 39 A) Aldefluor assay to quantify the percentage of aldefluor positive cells in TPC SLUG cl5 with respect to control cells B) mRNA fold induction of stemness markers in TPC-1 SLUG with respect to control cells. C) Quantification of sphere number by the formula (number of formed spheres/number of wells) x100 and of sphere size (mean diameter in μm) ($p<0.05$).

Similar data were obtained by enforced expression of SLUG in 850-5C cells (not shown). Interestingly, SNAIL overexpression was not capable of inducing EMT and stemness in TPC-1 cells (data not shown). Our data indicate that IL-8 stimulates SLUG expression, and ectopic expression of SLUG in TC cells mimics the effects of chronic IL-8 stimulation, suggesting that SLUG might be a critical mediator of IL-8-induced biological effects.

To confirm the link between IL-8 and SLUG in thyroid cancer, we performed a IHC on a panel of 28 human TCs including PTC, FTC and ATC, in order to evaluate the presence of IL-8 and SLUG. In accord with previously published data (Hardy RG et al. 2007, N Wang et al 2014), we found SLUG expression in thyroid carcinomas; as shown in *Table 5,* IL-8 expression correlated with the presence of lymph-node metastases (LMN) ($p = 0.026$), but not with other parameters.

	IL-8			
Histotype	**Negative**	**Low**	**High**	**P value**
Papillary Follicular Anaplastic	23(56,09%) 7(87,59%) 1(100%)	9(21,95) 1(12,5%) 0	9(21.95) 0 0	0,436
LNM				
Negative Positive	22(66.11%) 9(64.28%)	10(27,77%) 0	4(11.11%) 5(35.71%)	0,026
Size	Negative	Low	High	P value
T1 T2 T3	5(55,55%) 1(25%) 19(67,85%)	2(22,22%) 1(25%) 5(17,85%)	2(22,22%) 2(50%) 4(14,28%)	0,466

Table 5. This Table summarizes the correlation between IL-8 expression and clinicopathological parameters in a TMA of human TC specimens analysed by immunohistochemistry.

Moreover, as shown in *Table 6 ,* IL-8 expression correlated significantly with SLUG expression *(p value=0,012).*

	IL-8			
SLUG	**Negative**	**Low**	**High**	**P value**
Negative	17(73,91%)	6(26,08%)	0	
Low	6(60%)	0	4(40%)	0,012
High	5(38,46)	4(30,77)	4(30,77%)	

Table 6. IHC to shows a positive correlation between IL-8 and SLUG expression in a small series of human TCs.

5. DISCUSSION

Thyroid cancer (TC) is the most common endocrine malignancy and its incidence is increasing. TC includes well-differentiated (WDTC) follicular and papillary (FTC and PTC respectively), poorly differentiated (PDTC) and anaplastic thyroid carcinoma (ATC). Despite WDTC has an excellent prognosis, with more than 90% cured upon therapy, a small percentage (approx. 5%) of them become resistant to radioiodine therapy (radioactive iodine resistant-RAIR), the main treatment for these patients. RAIR neoplasias can hardly be cured. RAIR include, beyond WDTC, PDTC, and ATC.
The recognition that cancer progression is highly influenced by its interaction with stroma has lead to the concept that cancer management may be improved by therapeutic targeting of the tumor microenviroment (Hofmeister V et al. 2008). A full understanding of tumor-strome interaction may allow to identify novel therapeutical approaches for advanced TC, such as ATC or RAIR, and to overcome the issue of drug resistance during treatment.
We have previously demonstrated that MCs (MC) are recruited in thyroid cancer through tumor cell-derived VEGF-A. MC exhibit a pro-tumorigenic role in thyroid cancer. MC inhibition, by using sodium cromoglycate (Cromolyn), strongly reduces the growth of thyroid carcinoma xenografts in immunodeficient mice (Melillo et al., 2010). Thyroid cancer presents an intense MC infiltrate, whose density correlates with invasiveness. The increased motility and invasiveness of tumor cells are reminiscent of Epithelial-to-Mesenchymal Transition (EMT), an important step in tumor progression, responsible for the acquisition of invasive properties by tumor cells (Thiery JP 2003). EMT is a well characterized mechanism, through which epithelial cells trans-differentiate and acquire an mesenchymal and invasive phenotype. Here, we demonstrate that MC and their mediators enhance migratory and invasive ability of thyroid cancer cells through the EMT induction. In fact, MC Conditioned Medium (MC CM) treatment of thyroid cancer cells (Nthy-ori, TPC-1 and 850-5C) causes the acquisition of a fibroblast-like morphology, typical of the EMT program, induces the up-regulation of typical EMT markers (SLUG, SNAIL, ZEB-1, Vimentin) and down-regulation of E-Cadherin (a hallmark of epithelial cells), enhances cell migration, as demonstrated by scratch wound healing assays. Moreover, in accord with the evidence that EMT causes the loss of the epithelial cell polarity and the decrease of intercellular adhesion, we found that MC CM treatment of human thyroid cells causes Tight junction (TJ) destabilization and Claudin-1 down-regulation. These data, taken togheter, indicate that MC CM contains soluble factors that can induce or potentiate EMT of thyroid-transformed (Nthy-ori) or -cancer-derived (TPC-1 and 850-5C) cells.
We previously showed that MC, when co-cultured with thyroid cancer cell lines or treated with their conditioned media (tumor-educated MC, TEMC),

undergo activation and produce many inflammatory mediators. To identify the mediator(s) of MC CM-induced EMT in thyroid cancer cells, we evaluated the expression of MC mediators in TEMC by qRT-PCR Cytokine Array and by multicytokine ELISA Array analysis. We found that several MC cytokines and chemokines are highly up-regulated upon TC treatment. Among the different mediators, we found that IL-6, IL-8 and TNF-α are the strongest inducers of EMT, being IL-8 the most efficient, since it could recapitulate EMT with the same efficiency of MC CM. In fact, the blockade of IL-8, but not of IL-6 or TNF-α, by neutralizing antibodies inhibits the ability of MC CM to induce EMT, as assessed by wound healing, and this effect reverts by an excess of IL-8. The predominant role of IL-8 is probably due to higher levels of the IL-8 receptors CXCR1 and CXCR2 in comparison with TNFR and IL-6R.

The initiation and recurrence of tumors is believed to be strongly linked with the biology of cancer stem cell (CSCs). Recent studies have demonstrated that EMT plays a critical role not only in tumor metastasis but also in tumor recurrence and increasing evidences suggest that EMT is also associated with the generation of CSCs (Dejuan Kong et al. 2011). These cells are well known to possess self-renewal properties and are retained responsible for tumor initiation, chemo/radiation therapy resistance, recurrence and metastasis. Thus, it is important to identify factors that can induce/potentiate CSCs during cancer progression for the development of novel and targeted therapies for complete eradication of cancer (Weiyuan Fang et al. 2014).

According with previous observations (Todaro et al., 2010, Shimamura et al. 2014), screening of Nthy-ori, TPC-1 and 850-5C cells revealed, in each cell line, a distinct population with high aldefluor activity (a measure of ALDH activity, that increases in stem cells), that was more abundant in the 850-5C cells, derived from a human ATC. Consistently, 850-5C, but not Nthy-ori and TPC-1 cells, were capable to form spheres when coultured in low-adherence condition, a well characterized assay to evaluate stemness features.To confirm that thyrosphere are indeed enriched in stem cells, we compared the levels of stemness markers in 850-5C cells grown in adherence or in low-adherence conditions. 850-5C thyrospheres display higher mRNA levels of nestin, ABCG2 and ALDH, assessed by quantitative PCR in comparison to adherent cells. Moreover, FACS analysis showed that the levels of membrane-exposed CXCR1 and CXCR2 was higher in 850-5C thyrospheres than in adherent cells; $CXCR1^{high}$ thyrospheres were also positive for SOX2, a recognized stemness marker (data not shown). Interestingly, 850-5C thyrospheres were also enriched in IL-8 content with respect to adherent cells. These data indicate that the subpopulation of TC cells with stemess features is characterized by higher CXCR1/CXCR2-IL-8 expression with respect to the bulk population. Thus, not only thyroid cancer cells express the IL8 receptors CXCR1 and CXCR2, but also endogenous IL8; despite this, thyroid carcinoma cells are still responsive to IL8 stimulation. We showed, in fact, that exogenous treatment of TC cells with IL-8 causes, besides the induction of the EMT program, the expansion of

CSC population, as demonstrated by the increased number of $ALDH^{high}$ cells and the expression of CSC markers. IL-8 stimulation enhanced also thyrosphere-forming ability when TC cells were plated in ultra-low adherent conditions. Moreover, we show that endogenous IL-8 is involved in the manteinance of stemness in TC cells. First, endogenous IL-8 levels correlated with sphere-forming ability in a panel of TC cell lines (data not shown); accordingly, when we blocked IL8 signaling by using neutralizing antibodies directed to IL-8, CXCR1 or CXCR2, sphere forming capability is significantly decreased. Thus, the circuit IL8-CXCR1/CXCR2 mediates EMT and stemness of thyroid cancer cells.

We tryed to induce a stable EMT phenotype in TC cell lines by long-term stimulation (up to 60 days) with IL-8, that caused an increased expression of SLUG and Zeb1 and the acquisition of stemness features. However, this treatment was not able to stably induce these properties, as IL-8 withdrawal reverted this phenotype. In order to obtain TC cells chronically exposed to IL-8, we generated 850-5C overexpressing IL-8. These cells underwent EMT, as shown by mesenchymal-like morphology, increased expression levels of EMT markers and a higher capacity to repair wounds with respect to parental cells. IL-8 overexpressing clones also displayed a higher stemness potential with respect to control cells.

Athymic mice injected with $1x10^7$ 850-5C IL-8 adherent cells showed an increase in tumor growth in comparison to mice injected with parental or empty-vector transfected cells. 850-5C IL-8 exhibited a higher number of ki67 positive cells than 850-5C xenografts. However, this increase did not reach statistycal significance. 850-5C IL-8 xenografts displayed instead a significant increase in vascularization with respect to control tumors *(p value<0,001)*. Accordingly, IL-8 overexpressing cells did not display a faster growth in culture. On the other hand, IL-8 is a well recognized proangiogenic chemokine. Thus, the reason for the faster growth rate of 850-5C IL-8 with respect to control xenografts may be due, at least in part, to the augmented vessel density of the tumors.

We also demonstrated that 850-5C pBabe and 850-5C IL-8 thyrosphere-derived xenografts feature a higher tumorigenic potential with respect to adherent cells. In fact, both 850-5C pBabe and 850-5C IL-8 spheroid-derived, were able to form tumors when injected at $1x10^6$ cells; however, 850-5C IL-8 spheroid-derived tumors revealed a significantly shorter latency and a faster growth rate with respect to 850-5C pBabe spheroid-derived tumors *(p value<0.01)*. Moreover, 10/10 850-5C IL-8 spheroid-derived tumors xenografts efficiently grew in nude mice, while only 6/10 850-5C pBabe did so, indicating that tumor initiation was more efficient in 850-5C IL-8 than in control cells. In accord with our observations, OCT4 protein expression evaluated in a panel of 30 human PTCs was significantly associated with increased tumor size (T3, *p* value= 0.010), and correlated with Tryptase staining, a measure of MC density *(p value=0.025)*.

As we demonstrate that SLUG expression is maintained by long-term IL-8 treatment and 850-5C IL8 clones constitutively express high SLUG levels, we hypothesized it might be an important mediator of IL-8 biological activity and sufficient to induce EMT and stem-like features in TC cells. Thus, we ectopically expressed SLUG in TPC-1 cell line, that features low basal levels of this transcription factor. SLUG-overexpressing clones underwent EMT and acquired increased stemness features with respect to control cells. These results suggest that SLUG may be a critical mediator of IL-8-mediated biological effects. However, to finally demonstrate that SLUG is necessary for IL-8-mediated EMT and stemness induction in TC cells, we need to down-regulate SLUG expression by RNA interference. We generated SLUG-silenced clones and we are currently characterizing them for EMT and stemness.

The role of IL-8 in TC was also confirmed by analysing a limited number (n=28) of TC surgical samples by IHC for IL-8 and SLUG; we found that IL-8 expression correlates with the presence of lymph-node metastases (LMN) *(p value=0.026)*, but not with other clinicophatological parameters, and with SLUG expression *(p value =0,012).*

It has been demonstrated that many types of human carcinomas, including, breast, colon, cervical, gastric, lung, ovarian and medullary thyroid carcinoma express high levels of IL-8 relative to normal tissues Palena C et al. 2012 and Waugh DJ et al 2008). In addition, multiple clinical studies in MTC, melanoma, as well as breast, ovarian, prostate cancer have shown a direct correlation between serum IL-8 levels and disease progression (Broutin S et al 2011, Xie K. 2001). In recent years, it has also been demonstrated that a link exists between IL-8 and tumor stemness. The IL-8/CXCR1 axis has been well documented in breast CSCs. Populations of CSCs, characterized by elevated activity of aldehyde dehydrogenase, have been shown to express elevated levels of CXCR1 and, in turn, the addition of purified IL-8 to epithelial breast cancer cells increased the percentage of aldehyde dehydrogenase-positive cells, enhance the migration and invasiveness of CSCs in vitro (Charafe-Jauffret E et al 2009). Blockade of IL-8R activity via neutralizing antibodies or by utilizing the small-molecule inhibitor of IL-8R, repertaxin, decreased the breast CSC population both in vitro and in vivo (Ginestier C, et al. 2010), reinforcing the importance of the IL-8–IL-8R axis in breast CSCs. Additional studies conducted with primary colorectal cancer cells transfected with the stem cell-associated transcription factor OCT4 also showed that CSCs secrete higher levels of IL-8 and that neutralizing antibodies against IL-8 are able to inhibit tumorsphere formation along with the expression of the CSC markers CD133, CD44, SOX2, SNAIL and ABCG2, and decrease resistance to treatment with 5-fluorouracil (Chang CJ.et al 2011).

Searching for a mediator of IL-8 promoted biological effects, we noted that the chronic treatment of thyroid cancer cells with IL-8 causes a long term increase of SLUG, an EMT regulator and a stem cell marker, that plays also an important role in promoting tumor progression, invasion, chemoresistance and

radioresistance to cancer therapies (Shiori et al. 2006, Haslehurst AM et al 2012).

In conclusion, this study emphasize the importance of IL-8/CXCR1/CXCR2 axis in TC as an essential factor for the induction and maintenance of the mesenchymal and stem-like phenotype of aggressive, metastatic TC cells; our data indicate that IL-8 is both produced by stromal cells, such as MC, and produced in an autocrinous manner; therapeutic strategies for aggressive thyroid cancer are urgently needed and should target not only the bulk of cancer cells, but also CSCs, that are probably responsible for tumor progression and recurrence. Moreover these strategies can also target stromal cells such as MC. Thus, both endogenous and exogenous IL-8 contribute to EMT/stemness of TC cells. We cannot exclude that other stromal cells, including CAFs and immune cells, may be a source of IL-8. Accordingly, it has been recently shown that TAM are a major source of IL-8 in PTC. Moreover, TAMs represent a remarkable portion of stromal cells in ATC (Rayder et al. 2008, Caillou et al. 2011).

Targeting the IL-8/CXCR1/CXCR2 could be a promising startegy to target CSCs and to improve the outcome of difficult-to cure forms of TC, such as RAIR or ATC.

6. CONCLUSIONS

Despite recent evidences suggest the occurrence of EMT and stemness in human thyroid carcinomas, tumor microenvironment-derived mediators capable of provoking these features are unknown. Here, we identify MC as a critical component of tumoral stroma in TC and we show that IL-8 is the most important MC-mediator that induces EMT and stemness in thyroid cancer. This is possibly due to the induction of the EMT master transcription factor SLUG; accordingly the block of IL-8/CXCR1/CXCR2 axis in TC cells reverts EMT and stemness.

Current anticancer treatments are often able to destroy the bulk of a tumor but spare CSCs. Thus, relapse or tumor recurrence is not rare after a primary therapeutic response or initial induction of tumor remission. An ideal therapeutic strategy should not only kill differentiated cancer cells (as conventional therapy does) but should also specifically destroy thyroid CSCs simultaneously. Thus, the focus on thyroid CSCs has significant clinical implications for the development of novel therapeutical strategies for refractory thyroid cancer (such as RAIR and ATC).

Taken together, our results provide an interesting tool to uncover new avenues of therapeutic intervention with the potential to go beyond traditional anti-cancer approaches: because their involvement in tumorigenesis, migration and invasive ability through EMT induction and expansion of CSC population, MCs and their mediators are possible therapeutic targets for a novel strategy to treat thyroid cancer. In particular, we showed that IL-8/CXCR1/CXCR2 axis has a crucial role, placing neutralizing anti-IL-8 antibodies or small-molecule inhibitors of CXCR1/CXCR2 at the center of new and interesting therapeutic investigations.

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