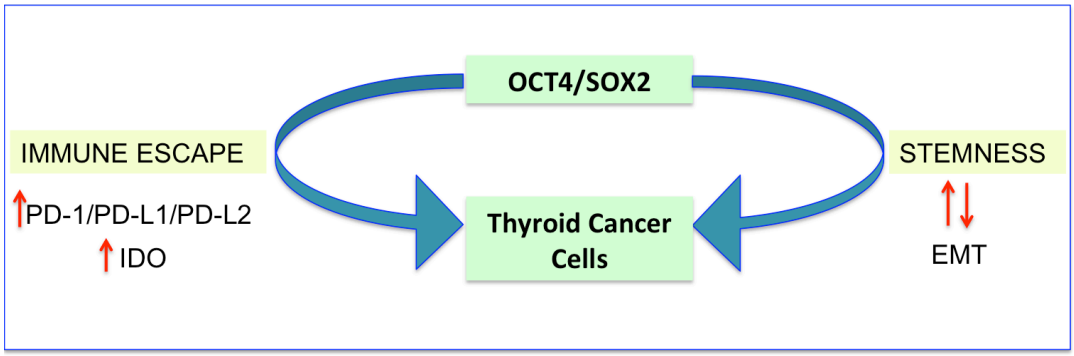
UNIVERSITY OF NAPLES FEDERICO II

PhD Program
MOLECULAR MEDICINE AND MEDICAL BIOTECHNOLOGY

XXX Cycle

"Stemness and Immunological Features of Thyroid Cancer Cells"



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Academic Year 2016/2017

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ABSTRACT

Cancer stem cells (CSCs) can initiate and maintain tumours and may drive metastasis, recurrence and resistance to anti-neoplastic therapies. Recent evidence suggests that CSCs possess immunomodulatory capabilities that may enable them to evade host anti-cancer immunity to promote tumorigenicity. CSC immunological functions include evasion from immune clearance, induction of clonal anergy or deletion, and activation of regulatory immune cells. We recently identified Interleukin-8 as a crucial factor that sustains the stemness features of thyroid cancer (TC) cells through the induction, among the others, of OCT4 and SOX2 transcription factors. We generated OCT4/SOX2 overexpressing TC cells and analysed their stemness and immunomodulatory properties. We confirmed that TC cell lines overexpressing OCT4/SOX2 showed, compared to control cells, an increase in stemness features. Accordingly, OCT4/SOX2 TC cells displayed increased tumour incidence and growth when injected at limiting number in mice compared to parental cells. Negative regulators of the immune system, including Programmed cell Death-Ligands 1 and 2 (PD-L1/PD-L2) and the enzyme Indoleamine 2,3-dioxygenase (IDO) are often "hijacked" by tumours to restrain the ability of the immune system to mount an effective anti-tumor response. Here, we show that OCT4/SOX2 expressing TC cells display increased PD1, PD-L1/2 and IDO expression both at mRNA and protein levels with respect to parental cells. Consistently, TC cells overexpressing OCT4 and SOX2, when co-cultured with lymphoid cells, caused a reduction of lymphocyte vitality compared to control cells. Thus, OCT4 and SOX2 are not only key regulators of stemness features but also of immunomodulatory properties of TC cells.

1.INTRODUCTION

1.1 Thyroid cancer

1.1 Thyroid cancer

In the embryo, thyroid develops from the neural crest cells and the primitive pharynx. Thyroid is a butterfly-shaped endocrine gland located on the trachea in the anterior part of the neck. This endocrine gland consists of two distinct lobes connected by an isthmus. Two types of cells reside inside the gland: follicular cells and parafollicular C cells. The follicular epithelial cells, are involved in iodine uptake and are able to synthetize the thyroid hormones. Between follicular cells reside parafollicular C cells that produce calcitonin hormone. Thyroid carcinoma (TC) is the most common endocrine tumour (1.0-1.5%). The incidence of thyroid tumours on all new cancer cases in USA is 3.4%; it has been estimated that in 2017 there will be 56.870 new cases of TC per year [1]. Ionizing radiations, genetic alterations, history of goitre, female gender and family history of thyroid disease, can be considered risk factors in the development of TC [1]. In Italy, TC represents the second most common tumour in women under 45 years of age [1]. Three principal pathological types of carcinoma arise from epithelial cells: papillary thyroid carcinomas (PTCs), follicular thyroid carcinomas (FTCs) and anaplastic thyroid carcinomas (ATCs). Instead, medullary thyroid carcinoma (MTC) arises from parafollicular cells. On the basis of the histological and clinical features we can divide thyroid carcinomas in well-differentiated, poorly-differentiated and undifferentiated/anaplastic types. Cancer is considered a multistep process in which the highly curable well-differentiated carcinomas (WDTCs) evolve into aggressive anaplastic carcinomas (ATCs). Poorly-differentiated carcinomas (PDTCs) assume an intermediate position in this progression concept [2]. WDTCs include papillary thyroid carcinomas (PTCs) and follicular thyroid carcinomas (FTCs), which are curable with radioiodine and exhibit a very good prognosis. PDTCs and ATCs are less common with respect to WDTCs and more aggressive exhibiting a bad prognosis. They are resistant to chemo- and radio-therapy and often develop metastases. PTCs represent 80% of all TC. PTCs usually present between 35 and 40 years of age and are three times more common in women than men; the peak of incidence is at 40-45 years. PTCs pathogenesis can be linked to radiation exposure; in fact 2-4% of the patients treated with ionizing radiations, mostly in the neck region, develops a differentiated thyroid carcinoma after about 20-30 years [3]. Generally PTCs spread into lymphatic blood vessels and present regional node metastases in a significant proportion of the cases (2%). It is known that FTCs occur in areas of low

iodine concentration and are considered more aggressive than PTCs. FTCs are only rarely associated with radiation exposure. World Health Organization (WHO) defines FTCs by the presence of capsular and/or vascular invasion. Moreover, PTCs show typical nuclear features while FTCs do not. In FTCs the diffusion into lymph nodes is uncommon, while the invasion into blood vessels within thyroid gland is common. Usually FTCs develop metastasis more likely into distant organs than into regional lymph nodes. PTCs and FTCs, are WDTCs, have an excellent prognosis. Therapy for both PTCs and FTCs consists in the complete removal of the gland followed by a metabolic treatment with Iodine-131 (^{131}I). The patients show a survival rate at 10 years of 90-98%.

The most aggressive type of thyroid cancer is the anaplastic thyroid cancer (ATCs). These carcinomas show highly abnormal cells and they are able to disseminate rapidly through other parts of the body **(Figure 1)**. ATCs represent only about 1% of all TCs and usually spread beyond the thyroid by direct local extension. The presence of metastasis to regional nodes is common, but often their presence is not clearly detectable because extensive soft tissue invasion is present. Unfortunately, no effective therapy is known for ATCs, since these tumours show a chemo- and radio-resistant behaviour. The prognosis is very unfavourable, and the patients often die one year after diagnosis [4]. Between WDTCs and ATCs there are the poorly differentiated PDTCs, rare aggressive thyroid tumours [5] **(Figure 1)**. PDTCs show variable morphology including 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 [6]. It is well known that the survival rate is poor among PDTCs patients than among those with WDTCs [7 8 9] **(Figure 1)**.

WDTCs generally have a favourable prognosis, but in ~5% of the cases they can progress to radioactive iodine-refractory (RAIR) tumours, which commonly lead to death within 5 years [10]. RAIR tumours show the loss of their ability to take up ^{131}I from the start of treatment or after previous evidence of uptake. This behaviour of malignant tissue is detected by a combination of imaging modalities, including an ^{131}I whole body scan showing at least one lesion that does not takes up ^{131}I, or clinical evidence that ^{131}I is no longer providing benefit to the patient [11]. Standard cytotoxic chemotherapy has limited efficacy, leading to the introduction in clinical trials of novel targeted therapies [10].

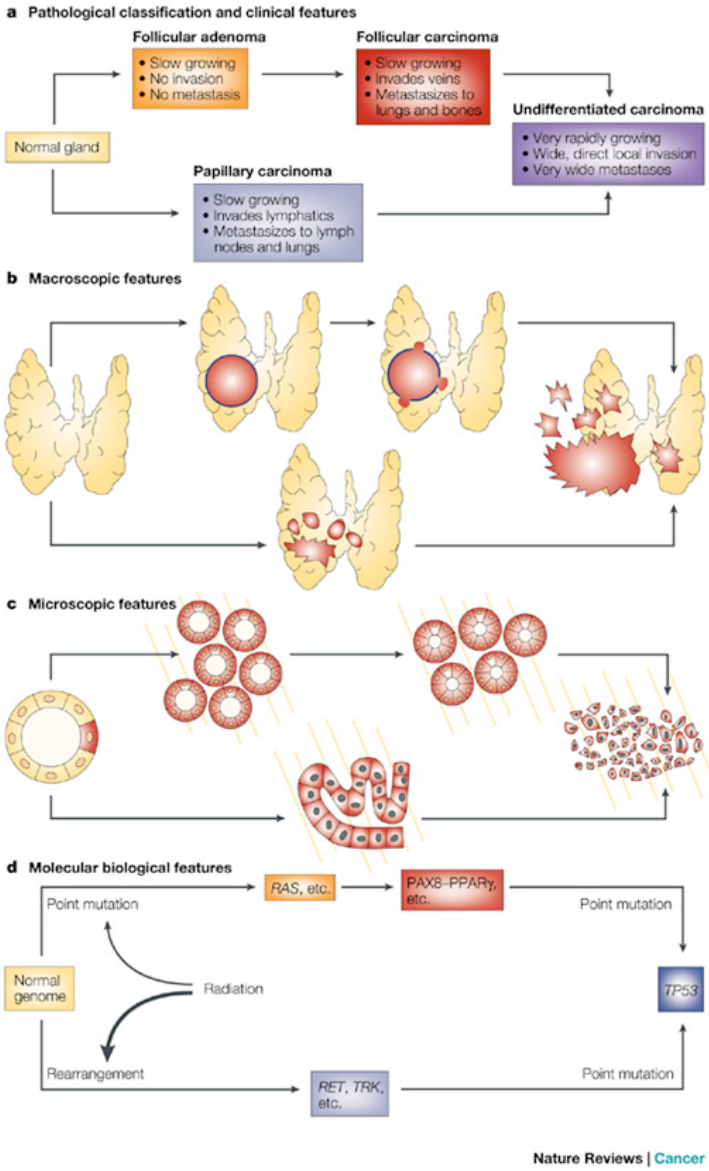

***Figure 1*:** Molecular features of thyroid tumours (Modified from Williams 2002).

Medullary thyroid carcinomas (MTCs) develop from parafollicular C cells of the thyroid and occur in about 5-7% of all TC. They 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, that shows MTC, pheochromocytoma and parathyroid adenoma; MEN2B that shows MTC, pheochromocytoma and additional tumours 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* (REarranged during Transfection) gene. MTCs tend to metastasize to lymph nodes and distant organs, and their treatment consists in surgical removal of the lesion. MTCs are fairly resistant to most chemotherapy.

1.2 Molecular genetics of thyroid tumors

1.2.1 PTCs – papillary thyroid carcinomas

PTCs include various histological subtypes: classical (CV), follicular (FV), tall cell (TCV) variant and other uncommon PTC variants. The development of PTCs is associated with different somatic mutations that include single nucleotide variants, small insertions and deletions, gene fusions and copy-number alterations. A recent TCGA study characterized the genomic landscape of human PTCs. Single nucleotide variants were identified in *MAPK*-related genes, including *BRAF, NRAS, HRAS* and *KRAS*: mutations in these genes are mutually exclusive [12]. *BRAF* mutations, observed in nearly 40% of PTCs, have been associated with a more aggressive course of disease [13]. The *RAS* family of small GTPases (G proteins) includes *KRAS*, *HRAS* and *NRAS*, all of which are membrane-bound GTP/GDP-binding molecules. These proteins transfer signals from activated receptors to pathways that lead to the synthesis of key proteins involved in cell growth, proliferation and survival. CV and TCV are mainly associated with *BRAF* mutations, while FV is associated with *RAS* mutations. 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 [14-16]. This mutation enhances *BRAF* activity through disruption of the auto-inhibited state of the kinase. Telomerase reverse transcriptase (*TERT*) promoter mutations, especially when co-existing with *BRAF* mutations, are associated with a poor prognosis in PTC. In addition, *EIF1AX* (eukaryotic translation initiation factor 1 A, X-linked) was identified as a novel cancer gene involved in PTC initiation and progression. Chromosomal rearrangements and translocations also contribute to PTC pathogenesis. Gene fusions are mutually exclusive with each other and with *BRAF, RAS,* and *EIF1AX* mutations. Often the fusions are associated with young age at diagnosis. Different genes were fused with *BRAF*, some of which supported *BRAF* signal with expression and conservation of its kinase domain (*MKRN1/BRAF*); in other cases, there was an alternative activating mechanism. *RET* rearrangements have been reported in sporadic and radiation-associated PTCs [17]. From a clinical point of view, PTCs could be classified into two molecular subtypes: $BRAF^{V600E}$-driven tumours (BVL-PTCs) and mutant *RAS*-driven tumours (RL-PTCs), these

groups showing different genomic, epigenomic and proteomic profiles. The first group is dominated by *BRAF*^{V600E} mutations, high levels of *MAPK* activation, loss of differentiation and high risk of recurrence; the second group is characterized by *RAS* mutations, relatively low levels of *MAPK* activation and dedifferentiation, and low risk of recurrence [18].

1.2.2 FTCs – follicular thyroid carcinomas

FTCs are the second most common type of thyroid malignancy, which accounts for 10-32% of cases of WDTCs [19]. The most frequent genetic alterations in FTCs are *RAS* mutations (*K, H* and *N*) and the *PAX8/PPARγ* rearrangement. The prognostic value of *RAS* mutations is still unclear, although some evidence suggests that *RAS*-mutated FTCs may be at risk for a poor prognosis or distant metastasis. Moreover, regarding the prognostic effect of the coexistence of *TERT* promoter and *RAS* mutations, it has been recently demonstrated that their coexistence might increase the risk of disease-specific mortality and recurrence in WDTCs, including both PTCs and FTCs [20].

1.2.3 PDTCs and ATCs – poorly differentiated thyroid carcinomas and anaplastic/undifferentiated thyroid carcinomas

Most PDTCs and ATCs are thought to arise from preexisting PTCs. In fact, they often share a driver mutation. ATCs and PDTCs show higher number of mutations with respect to PTCs. *BRAF*^{V600E} mutations are present in approximately 33% of PDTCs and 45% of ATCs, whereas *NRAS, HRAS*, or *KRAS* mutations occur in 28% and 24% of PDTCs and ATCs, respectively. *RAS* mutations are mutually exclusive with *BRAF* mutations and gene fusion. Also mutations in the *PI3K/AKT/mTOR* pathway are increased in ATCs (9%) and PDTCs (11%) with respect to PTCs (1%) [21]. These mutations occur in PTCs in a mutual exclusive manner with *BRAF* and *RAS*, while were closely related to *RAS* in PDTCs and ATCs. ATCs and PDTCs show an increase also in *TERT* promoter mutations with respect to PTCs [22]. They are significantly associated with *BRAF* or *RAS* mutations, consistently with the proposed mechanism by which mutations in the promoter, by generating novel consensus motif for the *ETS* family of transcription factors, promote *TERT* overexpression in cells with constitutive activation of *MAPK* signalling. *TP53* mutations were considered the principal hallmark of ATCs [23]. Recently, it has been demonstrated that p53 mutations were common in ATCs but very rare in PDTCs. In ATCs, chromosomal rearrangement were absent, while they were frequent in PDTCs. Gene rearrangements did not overlap with *BRAF, RAS, TSHR* mutations [12].

1.3 Cancer stem cells

1.3.1 Cancer stem cells properties

It is well known that stem cells in normal tissues are able of renewing themselves and simultaneously generating committed progenitor cells. Asymmetric cell division affords this process. Committed progenitor can eventually give rise to a different tissue with specific functions [24]. Recent data support the hypothesis that tumours contain a subpopulation of cells defined cancer stem cells (CSCs), which exhibit stem-like properties, such as the ability to self-renew, form tumourspheres in low adherence conditions, differentiate into various populations of cancer cells, and form new tumours in xenotransplantion experiments [25] [26] **(Figure 2)**. CSCs can originate both from the normal counterparts and from fully differentiated cells through a trans-differentiation process. CSCs were first identified in the hematopoietic system [27] more recently, however, they have also been discovered in solid tumours, including those arising in the breast, colon, brain, glioma, melanoma, prostate, lung, ovarian, renal cell and thyroid carcinomas [28-32].
According to the CSC model, CSCs drive tumour initiation and growth and these cells are considered to be a cause of tumour relapse and disease progression, perhaps through their resistance to therapy and metastatic potential. Therefore, CSCs are currently extensively studied in various cancers.

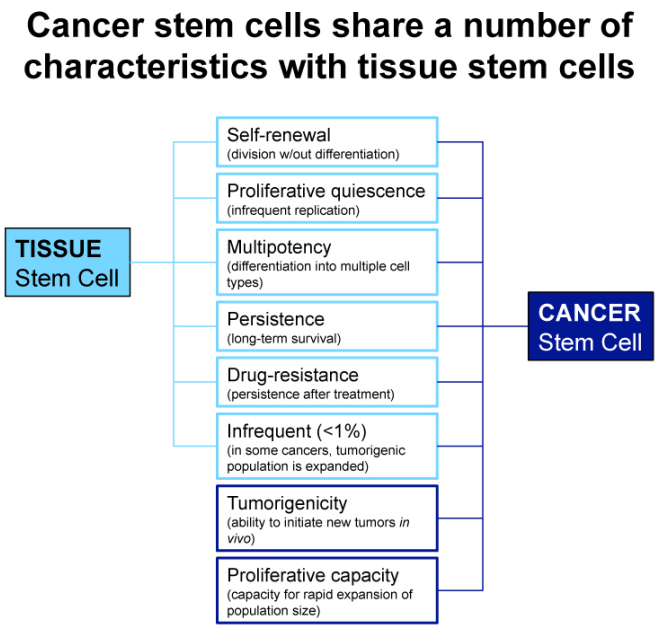

Figure 2: Characteristics of normal tissue stem cells and cancer stem cells.

1.3.2 Study and Characterization of CSCs

CSC and normal stem cells share various important properties: unlimited capacity for self-renewal, asymmetric division, the ability to differentiate into several cell lineages and the intrinsic resistance against cytotoxic therapies through drug efflux mechanisms and slow cell cycling. To study CSCs is important their identification. The "gold standard" method to identify CSCs is to isolate them using biomarkers specific for the CSC population (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 tumours should initiate tumour growth and give rise to secondary tumours. It is well accepted that there are several CSC markers in solid tumours (e.g. $CD44^{high}/CD24^{low}$ for breast cancer, $CD133^{+}$ for brain tumour and colon cancer, $CD44^{+}$ for gastric cancer). There are several canonical markers that CSCs share with normal stem cell SOX2 (SRY-related HMG-box gene 2), initially reported to be strongly linked with the inhibition of neuronal differentiation, has been shown to act as an important transcriptional factor to maintain the self-renewal capability of embryonic stem cells (ESCs). OCT4 (octamer-binding transcription factor 4), a member of the family of POU domain transcription factors that bind SOX2, represents also a key regulator essential for the pluripotency and self-renewal of ESCs. NANOG (Homeobox protein 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. ABCG2, widely expressed in stem cells, is also found to confer the "side population" phenotype. Side population phenotype is recognized as a universal marker of stem cells. This feature can be assessed by using a discrimination assay, a flow cytometry method used to detect stem cells based on the dye efflux properties of ABC transporters. Nestin is an intermediate filament protein that is known as a neural stem/progenitor cell marker. Increased aldehyde dehydrogenase (ALDH) activity is another hallmark of CSCs 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, is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome of breast cancer patients [33]. High ALDH1 activity has been used to define stem cell populations in many cancer types and has been described in primitive cells from multiple myeloma, acute myeloid leukemia, pancreatic, breast, and lung carcinomas [34].

1.3.3 Thyroid cancer stem-like cells

Recent studies in thyroid cancer (TC) suggest that histological appearance and biological behaviour often show heterogeneity, with cells exhibiting distinct proliferative and differential capacities [33 35].

The properties of thyroid CSCs remain not completely understood compared with that other of tumour types. A stem-like cell subpopulation, isolated from human TC specimens, featured growth in ultra-low adherence conditions *in vitro*, and cancer-initiating ability *in vivo* [32, 36-38]. Moreover, clinical observations have revealed that CSC marker frequency in TC is related to adverse outcomes [39]. Thus, CSCs have been indicated to play a crucial role in the malignant progression and therapeutic resistance of TC [40]. Various research groups observed that more differentiated TC cell populations acquire CSC properties through EMT [41-43]. These studies, therefore, raise the possibility that thyroid CSCs may arise from restricted progenitors or more differentiated cells that have acquired self-renewing capacity.

Various human thyroid cancer stem cell (TC SC) markers have been proposed to distinguish CSCs from the bulk of tumour cells [44 45]. However, the factors that specifically identify and regulate TC SCs are still undefined.

Multiple cytokine networks are crucial in regulating CSC activity. Among these, Interleukin-8 (IL-8/CXCL8) is a key cytochine to maintain CSC functions in various tumours [46]. IL-8 is a proinflammatory/chemoattractant cytokine, produced by leukocytes and other cell types (i.e., epithelial cells), that contributes to human cancer progression by sustaining cell proliferation, invasion, migration, angiogenesis and survival [47]. IL-8 belongs to the CXC chemokine family and exerts its biological effects through two G protein-coupled receptors, CXCR1 and CXCR2 [47]. We previously identified mast cell-derived IL-8 as a key paracrine factor that activates the epithelial-to-mesenchymal transition (EMT) and increases the stemness features of TC cells [43]. Consistently, previous studies demonstrated that the most common oncogenic drivers in TC (*RET/PTC, HRAS*^{V12} or *BRAF*^{V600E}) can activate an intrinsic inflammatory pathway responsible for the autocrine production of several chemokines/cytokines, including IL-8 [48 49]. We confirmed that IL-8 is expressed by human TC specimens and TC-derived cell lines. We showed that IL-8 production and CXCR1/2 expression are enriched in TC SCs with respect to the bulk of TC cells [50]. Thus, IL-8 can be provided by both TC stroma and epithelial cells. The IL-8/CXCR1 circuit is crucial for maintenance of the stemness features and tumour-initiating ability of TC cells [50].

1.3.4 CSC transcription factors

In many human cancers OCT4, NANOG, and SOX2 have been found to be up-regulated, including oral squamous cell carcinoma [51], prostate cancer [52], and breast cancer [53]. OCT4 has been shown to be up-regulated in additional

cancers, including murine Lewis lung carcinoma [54], bladder cancer [55], and seminoma [56]. NANOG and SOX2 have been found up-regulated in cases of human somatic tumours. The expression levels of OCT4, NANOG, and SOX2 mRNA transcripts, which are detected in tumour cells and CSC niches, are usually higher than those of non-tumour tissue. They are also more frequently overexpressed in poorly-differentiated tumours than in well-differentiated tumours [53]. In fact, the expression levels of pluripotent factors can decrease with the differentiation of the cells. The mechanism by which OCT4, SOX2 and NANOG act in CSCs is in part different in Embryonic Stem Cells (ESCs). Although CSC and ESC share the property of self-renewal, ESCs emphasize differentiation, whereas CSCs emphasize proliferation. OCT4, NANOG, and SOX2 together maintain the repression of lineage-specific differentiation in ESCs. However, in CSCs, the overexpression of OCT4, NANOG, and SOX2 modulates signalling pathways to inhibit apoptosis [57]. Consistently with the central role of OCT4 and SOX2 in other CSC models, we found that thyrospheres, enriched in stem-like cells, expressed significantly higher mRNA and protein levels for the stemness markers OCT4, SOX2 and NANOG than adherent cells. The expression of SOX2 and OCT4 was efficiently induced by IL-8 in TC cell lines. Furthermore, IL-8 was able to activate in TC cell lines the stemness reporter SORE6 [58] in which six repeats of a SOX2/OCT4 response element from the proximal human NANOG promoter [59] drives the expression of the mCherry reporter gene [50].

1.4 CSC and immune-surveillance

The immune system plays a key role in the surveillance against the tumours. Three main mechanisms are involved in this activity. First, elimination and suppression of viral infection are able to protect the host from virus-induced tumours. Second, the elimination of pathogens and resolution of inflammation can prevent the establishment of an inflammatory environment that promotes tumorigenesis [60]. Third, the immune system is able to identify and eliminate tumour cells on the basis of their expression of tumour–specific antigens or molecules induced by cellular stress [61]. The third process is identified to as tumour immune-surveillance, whereby the immune system recognizes cancerous and/or precancerous cells and eliminates them before they can evolve in a tumour. Despite tumour immune-surveillance, tumours develop in the presence of a functioning immune system [60].

In the interaction of host with tumour cells, three essential phases have been proposed: elimination, equilibrium and escape **(Figure 3)** [62]. In the first phase, the immune system detects and eliminates tumour cells. During elimination all tumour cells can be cleared to obtain complete elimination phase or only a portion of tumour cells can be eliminated to obtain an incomplete elimination phase. In the case of partial tumour elimination, a

temporary state of equilibrium can then develop between the immune system and the developing tumour. It is possible that during this period tumour cells either remain dormant or continue to evolve, accumulating further changes (such as DNA mutations or changes in gene expression) that can modulate the tumour-specific and stress-induced antigens. This process allows the immune system to exploit a selective pressure by eliminating susceptible tumour clones where possible.

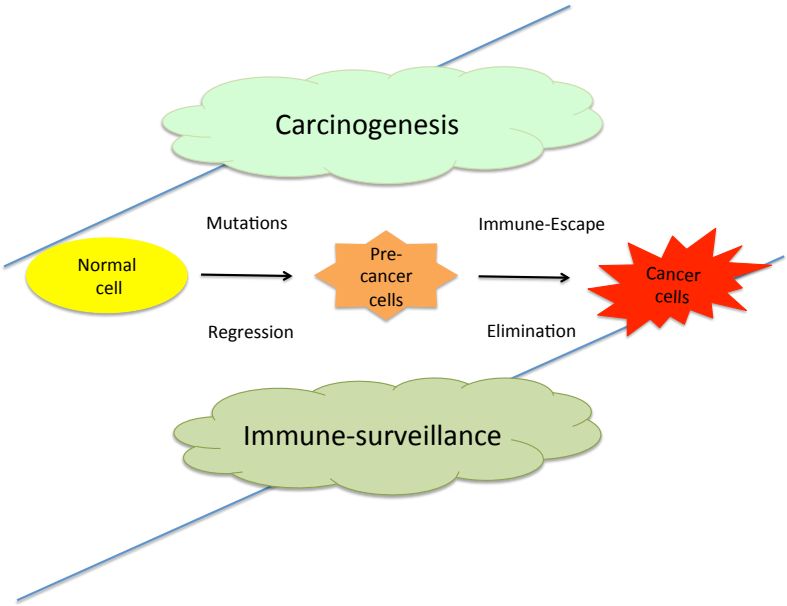

Figure 3: Immune system continually recognizes and eliminates cancerous cells. The mutated cells, when evolving in precancer and cancer cells, escape immune-surveillance by generating immunosuppressive mechanisms.

Using pressure selection mechanism, the immune system controls tumour progression, but sometimes, the immune response fails to completely eliminate the tumour selectioning tumour cell variants that are able to resist, avoid, or suppress the anti-tumour immune response, leading to the escape phase. During the escape phase, the immune system is not able to suppress tumour growth, and the tumours progressively emerge **(Figure 3)** [62].

The immune system consists of various cell types able to mount both innate and adaptive immunity against pathogens. As an important component of adaptive immunity, T cell activation requires three signals [63]. Signal 1 is acquired through the interaction between T cell receptor (TCR) and peptide-loaded major histocompatibility complex (MHC) molecules. Signal 2 is induced through the interaction between co-stimulatory molecules on antigen-presenting cells (APC) and their receptors on T cells, thus enhancing T cell activation. Signal 3 is mediated by cytokines, such as IL-2 to promote

T cell proliferation. Co-stimulatory molecules are the most well-studied molecules and fall into immunoglobulin super family members, including B7 family and tumour necrosis factor (TNF) family proteins. In order to limit immune response, co-inhibitory molecules are coopted. The immune inhibitory pathways is crucial for maintaining self-tolerance are hijacked from tumour cells to escape immune system control [64]. Immune system normally is able to remove cancer cells from our body. However, many patients develop immune tolerance to tumour cells through up-regulation of immune regulatory molecules, release of immune suppressive factors in the tumour microenvironment and/or recruitment of regulatory/suppressive cells that impede the function of other fully activated effector immune cells [65]. Cancer cells express many antigens that can be recognized and presented to T cells, leading to T cell activation and elimination of the tumour cells. This T cell immune response is modulated by negative regulatory molecules such as the immune checkpoint molecules such as PD-1, PD-L1, PD-L2, and IDO. Deregulation of these molecules in the tumour leads to tolerance of the tumour by immune system and cancer escape from surveillance [64]. It has been observed that inhibitory ligands and receptors are usually overexpressed in cancer cells or their microenvironment. Inhibition of these immune checkpoints releases the brakes on the immune system, resulting in antigen-specific-T-cell responses. Programmed Death-1 (PD-1) is a member of CD28 family expressed on T cells PD-1 inhibits their survival, proliferation and immune function through interaction with its ligands PD-L1 and PD-L2. Interactions between PD-1 and PD-L1 attenuate immune response and serve to protect tumour cells from cytotoxic T cells. Indoleamine 2,3-dioxygenase (IDO) is the first and rate-limiting enzyme of tryptophan catabolism through the kynurenine pathway, thus causing depletion of tryptophan which can cause halted growth of microbes as well as T cells. It has been shown that IDO permits tumour cells to escape the immune system by depletion of L-tryptophan in the microenvironment of cells and by production of the catabolic product kynurenine, which selectively impairs the growth and survival of T cells [64].

1.4.1 Immunomodulatory properties of cancer stem cells

Recent studies revealed that CSCs show immune-privileged phenotype with respect to normal stem cells [66]. At least this has been shown to be the case for two types of cancers: melanoma and glioma.

Melanoma CSCs have been identified as ATP-binding cassette sub-family B member 5 (ABCB5)-positive cells [67]. Specifically, the melanoma CSCs $ABCB5^+$ positive cells had significantly lower expression of MHC class I compared with the bulk of melanoma cells. In addition, $ABCB5^+$ CSCs show an increase in the PD-L1 expression by compared with the bulk ($ABCB5^-$)

melanoma cells. These evidences suggest a lower ability of CSCs to induce an immune response compared with the bulk of the tumour [67].

Like many other types of cancer, the glioma microenvironment becomes immune suppressive, thus functionally inhibiting or inducing apoptosis of primed $CD8^+$ cytotoxic T-cells [68]. In addition, there is increased recruitment of other immune suppressive cells like $FOXP3^+$ regulatory T-cells [69]. Importantly, several studies have shown that this suppressive effect is especially exerted by glioma CSCs [66]. Interestingly, when breast cancer cells were induced to go through EMT process under the influence of the transcriptional factor Snail, an EMT inducing gene, they promoted the escape of breast cancer cells from lysis mediated by $CD8^+$ cytotoxic T cells [70]. This further supports the role of CSCs in the immune escape of breast cancer. This effect is not limited to breast cancer but was also found to be relevant to melanoma. Melanoma cells induced to undergo EMT under the influence of Snail generated $FOXP3^+$ Treg cells and impaired the maturation of dendritic cells both *in vivo* and *in vitro* [71]. Collectively, the above data support that EMT, a process that enrich for CSCs, exhibits immunomodulatory properties.

2. AIM OF MY STUDY

The aggressive features observed in many tumours have been attributed to a specific cell population that exhibits stem cell-like properties, defined as cancer stem cells (CSCs). These features include tumorigenic activity, recurrence, resistance to chemo- and radio-therapy, capacity to form metastasis.

TC is not an exception in this regard, as a CSC population (TC SC) has been identified also in this type of cancer, based upon the stemness features of these cells, including the ability to form tumours in serial transplantation experiments. While in other cancer types the CSC population has been identified and isolated thanks to specific membrane markers, the TC SC population is still uncharacterized. Many reports indicate that TC cells acquire stemness features through the epithelial-to-mesenchymal transition (EMT) process. Thus, factors that promote EMT can also trigger stemness. According with this concept, we recently identified Interleukin-8 (IL-8/CXCL8) as a crucial paracrine/autocrine factor that induces EMT and increases stemness features of TC cells. In fact, IL-8 induced a persistent increase in the SLUG transcription factor, a master regulator of EMT. This, in turn, activated EMT and enhanced stem-like features of TC cells. In fact, we identified OCT4 and SOX2, among the others, as important genes whose mRNA and protein levels could be increased by IL-8 treatment, in a SLUG-dependent manner [43 50].

Here, we investigated the features of TC stem-like cells (TC SCs). To this aim, we set up various cellular systems with enhanced stemness features. We established TC cultures ectopically co-expressing the OCT4/SOX2 transcription factors. We also used SLUG- or IL-8-overexpressing/silenced TC cells that were previously developed and characterized in our laboratory. We analysed the stemness/malignant features of OCT4/SOX2 TC cells by evaluating their sphere-forming and self-renewal efficiency, their ability to proliferate, survive, migrate and form tumours into immunodeficient animal models at limiting numbers.

Moreover, we characterized the immunosuppressive features of different TC cell lines, derived from human TC of different histology (including PTCs and ATCs), by evaluating the levels of immunomodulatory molecules and the ability to suppress lymphocyte vitality. We also assessed the impact of stemness on this immune phenotype by comparing the immunosuppressive ability of parental TC cells with the same cells featuring enhanced stemness features, including OCT4/SOX2-, SLUG- and IL-8-transfected cells.

3. MATERIALS AND METHODS

3.1 Cell cultures

Nthy-ori (or Nthy-ori 3.1) are human SV-40 immortalized thyroid epithelial cells. TPC-1 (RET/PTC1), BcPAP (Braf ^{V600E} and P53^{D259Y}) are derived from human thyroid papillary carcinomas; 8505c (BRAF^{V600E} and P53^{R248G}), SW1736 (BRAF^{V600E}), FRO (Braf^{V600E}), and BHT-101 (BRAF^{V600E}), Cal62 (KRAS^{G12R}) are derived from anaplastic thyroid carcinomas. All these cell lines grew in DMEM (Dulbecco's Modified Eagle Medium), 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 5% CO_2 [50].

3.2 Generation of stable clones

To generate stable clones, 850-5C and TPC-1 cells at approximately 70% confluence were transfected with pBabe-puro empty vector or co-transfected with pBabe-puro and pMXs-hOCT4 (# 17964) and pMXs-hSOX2 vectors (# 17965) (Addgene) by electroporation (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 analysed for mRNA and protein expression and biological activity [50].

3.3 Sphere forming

For the spheroid-forming assay, cells were plated at 5 cells/well in ultra-low-attachment 96-well or in ultra-low-attachment 100 mm 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% CO_2 at 37°C. Spheres were counted and their diameters measured after 15 days.

3.4 Self-renewal and limiting dilution assay

For *in vitro* serial passaging, thyrospheres were collected by gentle centrifugation at 800 rpm for 5 min and dissociated mechanically. The dissociated cells were passed through 40 μm mesh filters (BD Falcon Cell Strainer, Franklin Lakes, NJ) to eliminate doublets or triplets. Single cells were plated at 5 cells/well on ultra-low attachment 96-well plates to generate second and third generations for self-renewal of thyrospheres [50].

The aim of Limiting Dilution Assay is to estimate the enrichment of stem

cells in a large population. This subpopulation represents the part of the cells that shows "active cell frequency". For limiting dilution assay [72] cells were plated in decreasing numbers at 1, 5, 10, and 50 cells/well. Cultures were maintained until day 15, when the number of wells containing spheres for each cell plating density (number of positive cultures) was recorded, calculated and plotted using online ELDA analysis program (Http://bioinf.wehi.edu.au/software/elda). ELDA indicated significant differences in stem cell frequencies between the 8505c pBabe and 8505c OCT4/SOX2 cells [50].

3.5 Migration assay

Migration assay was performed in a 48-well-modified Boyden chamber (Neuroprobe). Briefly, 30 µl of complete DMEM medium were applied in the lower compartment the Boyden chamber. 10 µm-thick uncoated nucleopore membrane (Neuroprobe) with a pore diameter of 8 µm, previously treated with Fibronectin (50 µg/ml Sigma), was applied on Boyden chamber. 2×10^5/ml 8505c OCT4/SOX2 and TPC-1 OCT4/SOX2 stable clones and relative controls were applied to the upper part of the membrane (10^6/ml). Cells were incubated for 8-10 hours at 37°C in 5% CO_2 atmosphere. The membrane were then carefully disassembled and fixed with methanol (100%). To check the migrated cells, we performed haematoxylin-eosin staining. The average number of migrated cells from 15 representative fields (three replicates per condition) was counted under a phase contrast microscope [43].

3.6 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 per1 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. cDNA synthesis was conducted as follows: random-primed first strand cDNAs 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 [73], RNase Inhibitor, dNTP (Deoxyribonucleotide triphosphate) and Reverse Transcriptase. 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 measuring 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 quantify 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 SYBRGreen 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, 8 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-(sample 1 ΔCt - sample 2 ΔCt), 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/cgibin/primer3/primer3_www.cgi and synthesized by Eurofins Genomics.
The employed primer sequences are reported in the table below:

Table 1

Name	Forward 5'→ 3'	Reverse5'→ 3'
hGAPDH	ctgccactgaaaaggaggag	ttggcactccttgggttatc
hSOX2	gcgaaccatctctgtggtct	aaaatggaaagttgggatcg
hOCT4	agcaaaacccggaggagt	ccacatcggcctgtgtatatc
hABCG2	tggatttacggctttgcagc	tcctgttgcattgagtcctgg
hNANOG	tacctcagcctccagcagat	Ttgctattcttcggccagtt
hALDH	gcaactgaggaggagctctg	Ttcgattaaatcagccaacttgt
hNestin	gaaacagccatagagggcaaa	Tggtttccagagtcttcagtga
hPD-1	cgcccttgtgctgatcctg	Tgctggtagtggtacatctcc
hPDL-1	agatgtgaaattgcaggatgcagg	Caattccaagagagaggagaagct
hPD-L2	atgatcttcctcctgctaat	Tcagatagcactgttcacttccctc
hIDO	actgtgtcctggcaaactggaag	Aagctgcgatttccaccaatagag
hTGF-β	tggcgttaccttggtaacc	Ggtgttgagccctttccag

3.7 Cytofluorimetric analysis

Briefly, cells were incubated (30 min at 4°C) with specific or isotype control Abs. When necessary, we performed cell membrane permeabilization using the Cytofix/Cytoperm kit (BD Biosciences, Canada). Primary antibodies used were reported in the table below:

Table 2

Description	
Anti human IDO efuor 660	eBioscience™
Anti human CD279 (anti-PD-1)	eBioscience™
Anti human 274 biotin (anti PD-L1)	eBioscience™
Anti human SOX2 antibody	Abcam
Anti human OCT4 antibody	Abcam
Anti human CD273 PE (anti PD-L2)	Miltenyi Biotec
Anti mouse CD45-PE	Miltenyi Biotec
Anti mouse CD8a-PerCP-Vio700	Miltenyi Biotec
Anti mouse CD4-PerCP-Vio700	Miltenyi Biotec
Anti mouse CD3e-PerCP-Vio700	Miltenyi Biotec

Cells were analyzed with a FACS Calibur cytofluorimeter using Cell Quest software (BD Biosciences, Canada). A total of 10^4 events for each sample were acquired in all cytofluorimetric analyses. Thyroids from C57BL/6 and C57BL/6 TG-TRK mice were subjected to mechanical disaggregation (GentleMACS™ Dissociator by Miltenyi Biotec). Cells were centrifuged for 5 min at 1200 rpm, resuspended in PBS and then used for FACS analysis [43].

3.8 BrdU Incorporation

Bromodeoxyuridine (5-bromo-2'-deoxyuridine, BrdU) is a synthetic nucleoside analogue of thymidine. BrdU is used to detect proliferating cells. BrdU can be incorporated into the newly synthesized DNA of replicating cells (during the S phase of the cell cycle), substituting for thymidine during DNA replication. Cells were grown in 60 mm plates until 70% of confluence. After an overnight starvation, BrdU was added at a final concentration of 30 μM and the cells were incubated for 30-60 min. After this, the BrdU was removed and cells were detached from the plates and permeabilized. The pellet was resuspended in 0.3 ml PBS, and incubated with anti-BrdU monoclonal antibody in the dark for 1h. At this point, cells were washed, incubated with fluorochrome-labeled secondary antibody, and analysed with FACS as above mentioned.

3.9 Propidium Exclusion assay

To evaluate cell viability, we used Propidium Exclusion assay. Propidium iodide is a non-permeant membrane dye that is generally excluded from viable cells. PI binds to double stranded DNA by intercalating between base pairs. PI is excited at 488 nm and emits at a maximum wavelength of 617 nm. In order to evaluate cell viability, cells were plated on 60 mm plates, serum deprived for 12 hours, harvested (including dead floating cells in the medium) and aliquoted up to 1 x 10^6 cells/1000 μL into FACS tubes. Cell pellets were washed 2X in PBS and resuspended in 100 μL of Flow Cytometry Staining Buffer in 2μg/mL PI. Cells were gently mixed and incubated for 1 minute in the dark. To adjust flow cytometer settings for PI, 5 - 10 μL of PI staining solution to a control tube of otherwise unstained cells was added. Determination of PI fluorescence (using the FL-2 channel) was performed with a FACS Calibur™ instrument.

3.10 Luciferase assay

To verify the activation of stemness in TC cells, we used the Stem Cell Reporter Array, a commercial array that allows to test the activation of Stem Cell-related signalling pathways by using luciferase assays in a 96-well cell culture plate. Each Cignal Reporter Assay (OCT4, Nanog, KLF4, SOX2, Myc, Hedgehog, Notch, Wnt, Pax6, MEF2) consists of a mixture of a pathway-focused transcription factor-responsive firefly luciferase construct and a constitutively expressing Renilla luciferase construct. 1x10^4 cells/well were added together with retrotrasfection reagent and then treated or not with IL-8. After 24 hours of treatment, luciferase activity was measured. To quantify luminescence, we used a dual-luciferase assay (Promega). According to standard protocol for Dual-Glo® Stop & Glo®, cells were

removed from the incubator and Renilla and Firefly luminescence was measured.

3.11 Purification and vitality determination of peripheral blood lymphocytes

Lymphocytes were purified from the peripheral blood of healthy volunteers, negative for HIV-1, HIV-2, hepatitis C virus, and hepatitis B virus Abs. Buffy coat cell packs were provided by the Immunohematology Service (University of Naples Federico II). Informed consent, according to the guidelines of University of Naples Federico II institutional review board for the use of human in research, was obtained. Cells were reconstituted in PBS containing 0.5 g/L HAS and 3.42 g/L sodium citrate and were layered onto histopaque1077 (Sigma Aldrich) and mononuclear cells were collected at the interface. Lymphocytes were further purified to wear homogeneity (95% - 98%) by depleting monocytes using CD14 microbeads (Miltenyi biotec). The magnetically labeled by retaining them on a MACS column in the magnetic field of the MidiMACS (Miltenyi biotec).

3.12 Xenografts in Mice

To evaluate cell tumorigenic potential, xenograft experiments were conducted. $1x10^6$ 8505c parental or OCT4/SOX2 cells were injected into dorsal portion of 4-week-old mice (10 CD1 nu/nu mice per group). Tumour diameters were measured at regular intervals with a caliper. Tumor volumes were calculated with the formula: $V=(A*B^2)/2$ (A=axial diameter; B=rotational diameter). This study was conducted in accordance with Italian regulations for experimentation on animals.

4.Results

4. 1 IL-8 stimulation induces stemness in TC cell lines

To identify stemness-related gene induction upon IL-8 treatment, we used a stem cell reporter assay, which allows to define the activity of several promoter of genes linked to stemness in response to different stimuli. To this aim, 8505c cells, derived from human ATC, were selected. These cells express the $BRAF^{V600E}$ mutant protein. Reverse transfection was performed into a multiwell plate containing the luciferase reporter gene under the transcriptional control of a basal promoter element joined to tandem repeats of specific stemness-related transcriptional response elements **(Figure 4)**.
Upon transfection, 8505c cells were incubated for 48h in the presence of the indicated plasmids. In the last 24 hours, cells were stimulated with IL-8 (100 ng/ml) or left untreated. At the end of the experiments, luciferase activity was detected by luminescence measurements, as described in materials and methods. As shown in **Figure 4**, IL-8 was able to significantly induce the activation of various promoters, including OCT4, NANOG, KLF4, SOX2, MYC, HEDGEHOG, WNT, and , to a lesser extent, of MEF2, PAX6 and NOTCH. These results indicate that IL-8 is able to induce and sustain stemness of TC cells, and confirmed our previous observation that OCT4 and SOX2 could be induced upon IL-8 treatment.

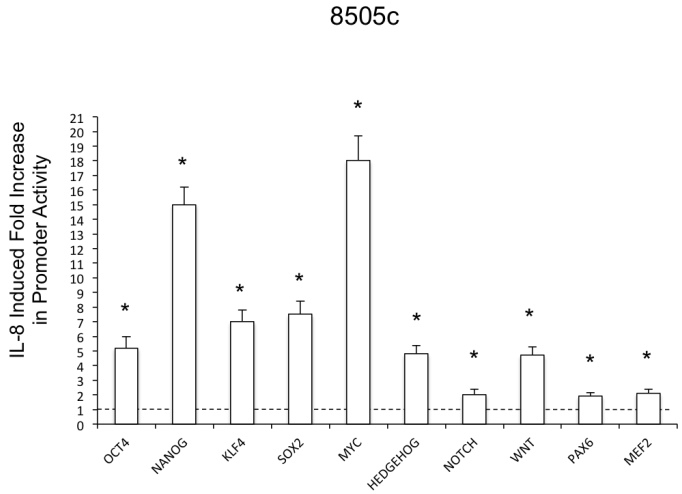

Figure 4: 8505c cells, derived from an human anaplastic thyroid carcinoma (ATC), were used for reverse transfection in a multiwell plate containing the indicated reporter vectors, and promoter activity was evaluated both in the presence and in the absence of IL-8 treatment (100 ng/ml for 24 h). Promoter activity of not-treated cells is represented by the dotted line. Mean ± SEM of three experiments. *, $p<0.05$ compared to the relative not-treated control.

4.1.1 Generation and functional characterization of OCT4/SOX2 overexpressing TC cells

We had shown that IL-8 treatment enhanced OCT4 and SOX2 gene expression in TC cells [43 50]. Furthemore OCT4 and SOX2 resulted strongly enriched in TC SCs [43 50]. Moreover, IL-8 treatment caused the activation of OCT4/SOX2-dependent promoters (**Figure 4**). Thus, OCT4 and SOX2 represent also in TC cells critical mediators of stemness. In order to obtain a stem-like population to study properties and functions of TC SCs, and to define the role of OCT4 and SOX2 in TC, we generated TC cells (8505c and TPC-1) overexpressing the two transcriptional factors. TPC-1 cells derive from a human PTC carrying a RET/PTC1 rearrangement.

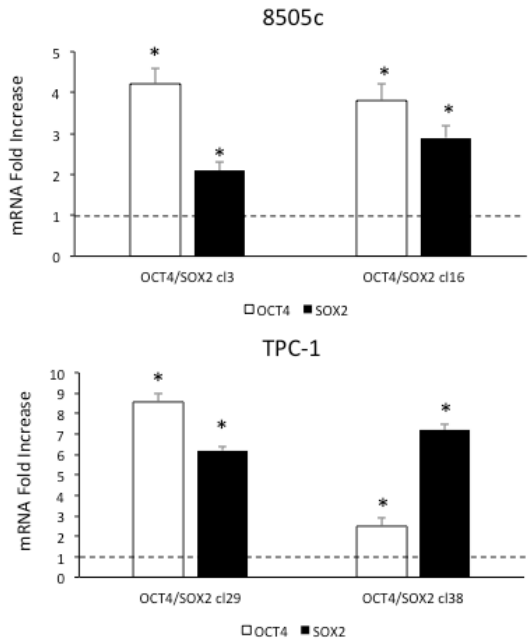

***Figure 5**:* mRNA fold increase of OCT4 and SOX2 in 8505c or TPC-1 cells transfected with both OCT4 and SOX2 expressing vectors compared to the expression levels of empty vector (pBABE) transfected cells (dotted line). Mean ± SEM of three independent evaluations. *, $p<0.05$ compared to empty vector transfected cells.

To this aim, 8505c and TPC-1 cells were transfected with OCT4 and SOX2 expression vectors. Transfected cells were subjected to puromycin selection. As a control, we also generated parental cells expressing empty vector (pBABE). Antibiotic-resistant clones were collected, expanded and successively analysed for OCT4 and SOX2 expression levels by quantitative PCR and cytofluorimetric analysis. As shown in **Figure 5**, two clones co-expressing ectopic OCT4 and SOX2 were selected for each cell line for

further experiments. OCT4 and SOX2 protein expression levels were evaluated by FACS analysis in the same clones (**Figure 6**). Thus, we obtained 8505c and TPC-1 cells featuring ectopic expression of both the OCT4 and SOX2 transcriptional factors.

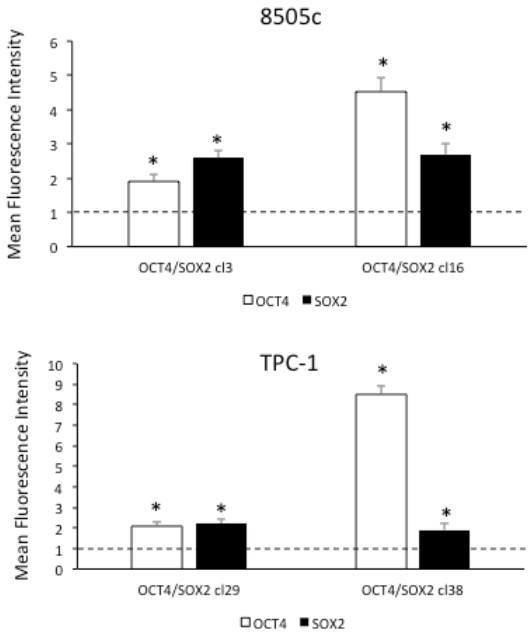

Figure 6: Mean Fluorescence Intensity evaluated by FACS analysis of OCT4 and SOX2 in 8505c or TPC-1 cells transfected with both OCT4 and SOX2 expressing vectors compared to the expression levels of empty vector transfected cells (dotted line). Mean ± SEM of three independent evaluations.*, $p<0.05$ compared to empty vector transfected cells.

The selected TPC-1 OCT4/SOX2 and 8505c OCT4/SOX2 cells were characterized for their growth and viability features. To this aim, we first evaluated the growth rate of these cells by performing growth curves in complete medium (DMEM - 10% FBS). These experiments showed that the growth capacity of both 8505c and TPC-1 OCT4/SOX2 clones was significantly higher than that of control cells (**Figure 7**). In line with these results, OCT4/SOX2-overexpressing 8505c or TPC-1 cells displayed a significant increase in DNA synthesis rate, as assessed by BrdU incorporation assay (**Figure 8**). In order to evaluate cell viability/apoptosis under stress conditions, we used Propidium Iodide [3] exclusion assay. Cells were plated, serum deprived for 24 hours, and then stained with PI. **Figure 9** shows representative FACS analyses demonstrating that OCT4/SOX2 overexpressing clones displayed increased cell death compared to control cells upon serum starvation.

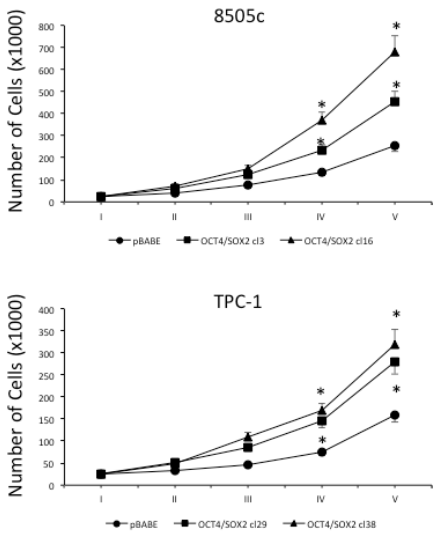

***Figure 7**:* Growth curves in 10% FBS of 8505c or TPC-1 transfected with the empty vector (pBABE) and of two clones overexpressing OCT4 and SOX2. Mean ± SEM of triplicate determinations. *, $p<0.05$ compared to empty vector transfected cells.

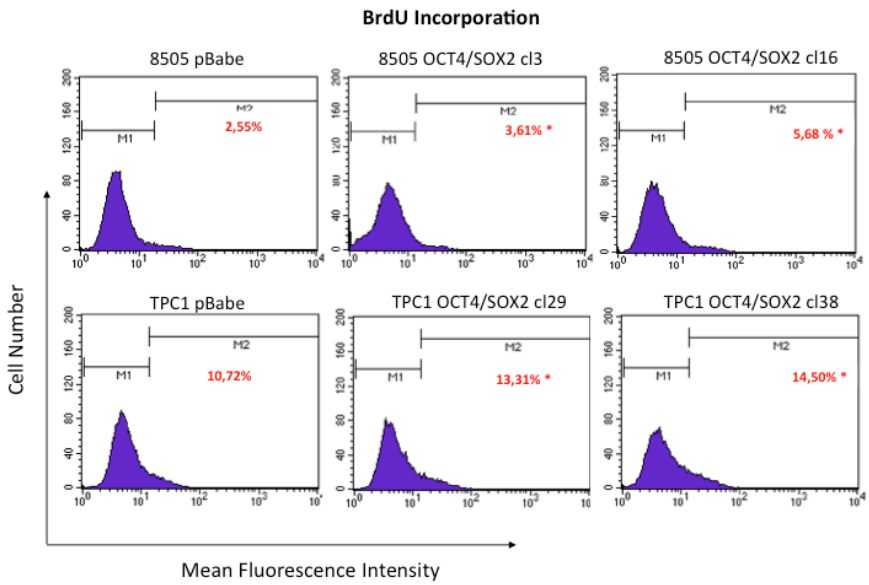

Figure 8: BrdU Incorporation evaluated by FACS analysis of 8505c or TPC-1 transfected with the empty vector (pBABE) and of two clones overexpressing OCT4 and SOX2. A representative experiment is shown. *, $p<0.05$ compared to empty vector transfected cells.

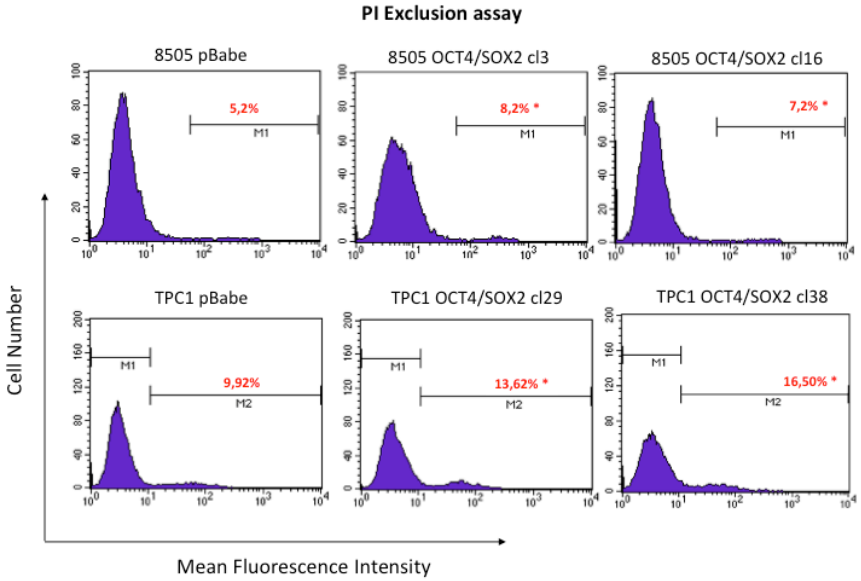

Figure 9: Percentage of dead cells assessed by FACS analysis through Propidium Iodide [3] staining of 8505c or TPC-1 transfected with the empty vector (pBABE) and of two clones overexpressing OCT4 and SOX2. A representative experiment is shown.*, $p<0.05$ compared to empty vector transfected cells.

These apparently contrasting results could be explained with the observation that some of the genes activated during reprogramming can cause detrimental effects including apoptosis and/or senescence [74]. It has been in fact demonstrated that the pro-apoptotic factor BAX is upregulated in response to the expression of OCT4, SOX2 and KLF4, and that the expression of the antiapoptotic BAX-antagonist molecule BCL2 results in enhanced reprogramming efficiency of iPSCs (induced Pluripotent Stem Cells) [75].
We then asked whether OCT4 and SOX2 overexpression could cause a more aggressive phenotype of TC cells. To this aim, we evaluated their capacity to migrate. Both 8505c and TPC-1 OCT4/SOX2 cells showed a significant increase in their migratory potential compared to control cells **(Figure 10)**.

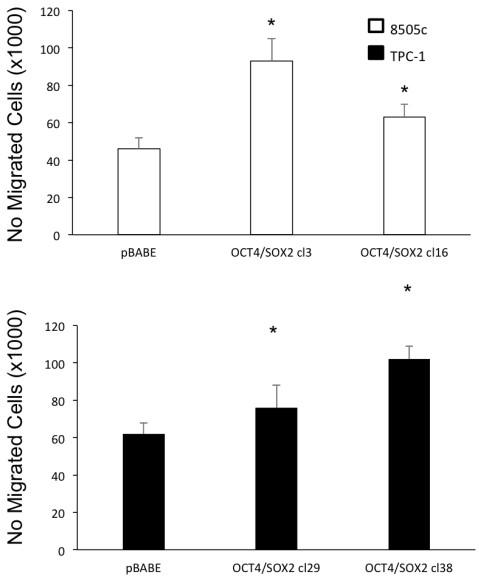

Figure 10: Number of migrated cells evaluated using a Boyden Chamber. 8505c or TPC-1 transfected with the empty vector (pBABE) and two clones overexpressing OCT4 and SOX2 were used. Mean ± SEM of three experiments.*, $p<0.05$ compared to empty vector transfected cells.

In conclusion, the enforced expression of OCT4/SOX2 in both PTC and ATC cell lines cells triggers a complex phenotype, characterized by enhanced DNA synthesis and proliferation, increased apoptotic rate in stress conditions, and enhanced migratory potential.

4.1.2 Stemness features of OCT4/SOX2 expressing TC cells

To define the stemness potential of OCT4/SOX2 overexpressing TC cells, we used different assays. In general, stemness features can be evaluated in cultured cells by checking the levels of stemness-related mRNAs and proteins, by evaluating the ability to grow in low-adherence conditions (sphere-forming, limiting dilution and self-renewal assays), and by measuring tumorigenic activity. Thus, we first evaluated the expression levels of mRNAs encoding for various stemness markers (NANOG, ABCG2, ALDH and NESTIN) by real-time PCR in TPC-1 OCT4/ SOX2, 8505c OCT4/SOX2 and in the respective control cells grown in adherence. As shown in **Figure 11A,** OCT4/SOX2 TC cells display a statistically significant increase in the mRNAs for stemness markers compared to control cells. Moreover, we investigated the hypothesis that OCT4/SOX2 overexpression can modulate the mRNA expression of EMT markers. Using real-time PCR we demonstrated that the expression level of three different EMT markers (SNAIL, SLUG and ZEB1) significantly increases compared to control cells **(Figure 11B)**.

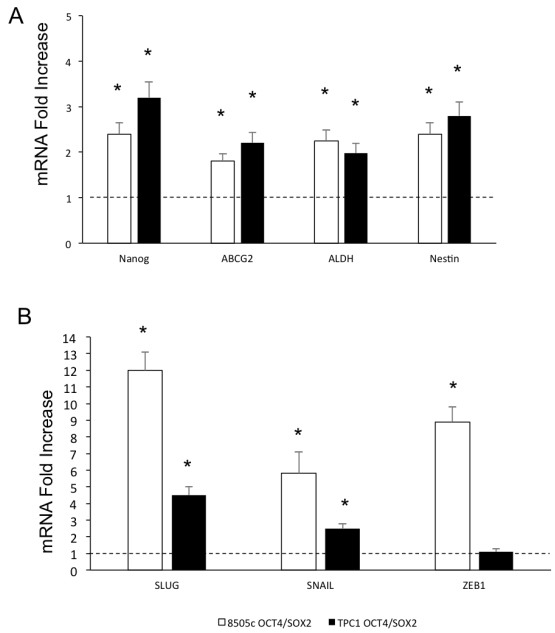

Figure 11A: mRNA fold increase of stemness markers (Nanog, ABCG2, ALDH, Nestin) in 8505c OCT4/SOX2 or TPC-1 cells OCT4/SOX2 compared to the expression levels of empty vector-transfected cells (dotted line). Mean ± SEM of three independent evaluations. *, $p<0.05$ compared to empty vector transfected cells.

11B: mRNA fold increase of EMT markers (SLUG, SNAIL, and ZEB1) in 8505c OCT4/SOX2 or TPC-1 cells OCT4/SOX2 compared to the expression levels of empty vector-transfected cells (dotted line). Mean ± SEM of three independent evaluations.*, $p<0.05$ compared to empty vector transfected cells.

One of the more popular methods to isolate and characterize CSCs and to assess their stemness potential *in vitro* is based on the capacity of these cells to grow in low-adherent conditions is the sphere formation assay. The inhibition of adhesion, in fact, induces cell death through anoikis in nonmalignant and differentiated epithelial cells. On the other hand, under the same culture conditions, undifferentiated/stem-like tumour cells proliferate and grow as floating clusters termed tumorspheres. Tumorsphere-forming efficiency generally correlates with stemness features [76]. By using this assay, we observed that OCT4/SOX2 cells displayed, compared to controls, an increased sphere-forming efficiency, as assessed by both sphere number and diameter **(Figure 12)**. Another classical feature of CSC is the ability to self-renew. Self-renewal ability can be evaluated both *in vivo* and *in vitro.* To evaluate self-renewal *in vivo*, putative CSCs are xenotransplanted into immunodeficient mice to allow tumour formation, and long-term tumour-propagating capacity is measured. To evaluate self-renewal *in vitro,* tumourspheres are serially passaged for few generations, and sphere-forming efficiency is measured at each generation.

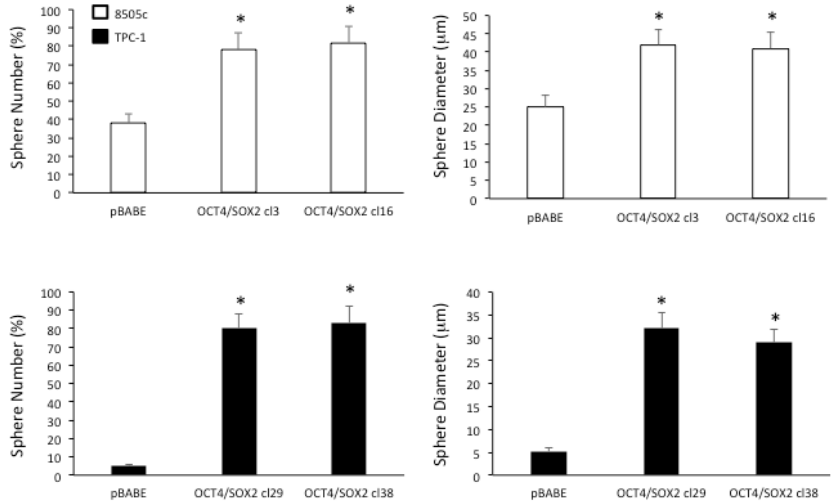

Figure 12: Sphere-forming efficiency, assessed as both sphere number and diameter (left), of 8505c or TPC-1 transfected with the empty vector (pBABE) and of two clones overexpressing OCT4 and SOX2. Mean ± SEM of three experiments. *, $p<0.05$ compared to empty vector transfected cells.

In synthesis, self-renewal measures the ratio of symmetrical/asymmetrical cell divisions. Thus, in order to evaluate whether OCT4 and SOX2 can increase the self-renewal ability of TC cells, we performed a sphere-forming assay across generations. **Figure 13** shows that 8505c empty vector-transfected cells (pBABE) are able to form spheres until second generation (F2) albeit with reduced efficiency with respect to F1. On the contrary, two OCT4/SOX2 overexpressing clones formed spheres until third generation.

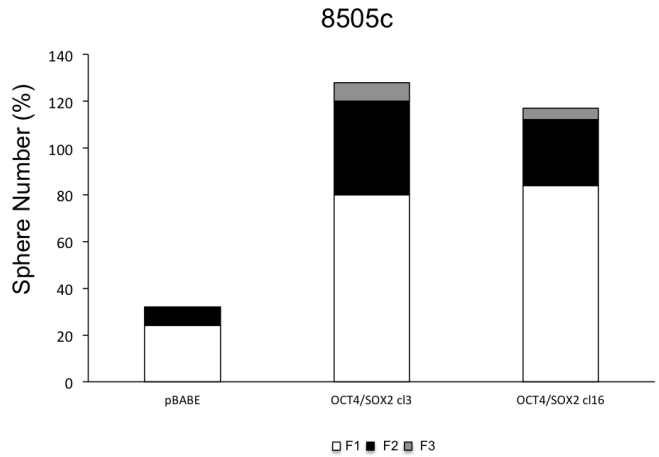

Figure 13: Self-renewal efficiency of 8505c cells transfected with the empty vector (pBABE) and of two clones overexpressing OCT4 and SOX2. *, $p<0.05$ compared to empty vector transfected cells.

Moreover, the sphere-forming efficiency of OCT4/SOX2 clones was significantly higher than that of parental cells at F1 and F2 generations.
To assess the role of OCT4 and SOX2 on the clonogenic capacity of TC SCs, we performed a limiting dilution assay on 8505c pBabe and on 8505c OCT4/SOX2 cells. We demonstrated that these two stemness factors significantly increased the clonogenic potential of 8505c (**Figure 14**). Collectively, these results confirmed that OCT4 and SOX2 transcriptional factors represent key regulators in the induction and promotion of TC cell stemness.

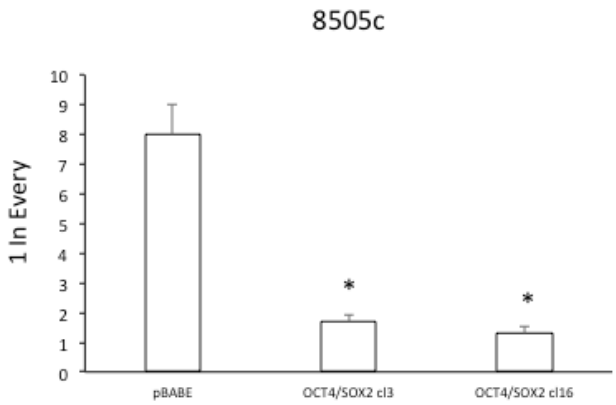

Figure 14: Extreme limiting dilution assay of 8505c cells transfected with the empty vector (pBABE) and of two clones overexpressing OCT4 and SOX2. The graph presents the clonogenic potential of each cell line. Mean ± SEM of three experiments. *, $p<0.05$ compared to empty vector transfected cells.

One of the features of CSCs is the ability to initiate tumours in xenograft experiments. Thus, to evaluate the tumourigenic activity of 8505c pBABE and 8505c OCT4/SOX2 cells, we performed xenotransplantation experiments. To this aim, two groups of 10 mice (CD1 Nu/Nu, females), were subcutaneously injected with $1x10^6$ cells, and tumour growth was measured weekly. $1x10^6$ cells represent for 8505c pBabe cells the limiting number whit which tumour formation can be detected. We found that OCT4/SOX2 overexpressing clones displayed higher tumorigenic potential with respect to the control 8505c cells. In fact, while the 8505c OCT4/SOX2 cells xenotransplanted into nude mice exhibited a tumour incidence of 50%, the 8505c pBABE tumour-forming efficiency was 16.6%. In addition, the tumours formed by 8505c OCT4/SOX2 clones were larger than those formed by control cells at the end of the experiments (**Table 3**).

8505c

	Tumor Incidence (%)	Tumor Volume (cm^3)
pBABE	16.6	0.001 ± 0.0002 *
OCT4/SOX2 cl16	50	0.08 ± 0.009 *

Table 3*:* Tumor incidence (%) and tumor volume (cm^3) of 8505c pBABE and of 8505c OCT4/SOX2 cl16 cell xenografts. *, $p<0.05$ compared to empty vector transfected cells.

4.2 Imunosuppressive phenotype of OCT4/SOX2-expressing TC cells

It is well known that CSCs are considered responsible of the aggressiveness of many types of tumours. One of the possible reason of such behaviour might be the ability of CSCs to evade the anti-cancer immune surveillance. To verify whether this is also the case for TC cells, we correlated stemnness features of TC cells with their immunosuppressive properties. To this aim, we evaluated the expression levels of various immunomodulatory molecules in TC cell lines overexpressing or not OCT4/SOX2 by real-time RT-PCR and FACS analyses. These experiments demonstrated that, among various immunosuppressive molecules, IDO, PD-L1, PD-L2 and TGF-β were enriched in OCT4/SOX2 8505c and OCT4/SOX2 TPC-1 cells compared to controls (**Figure 15**).

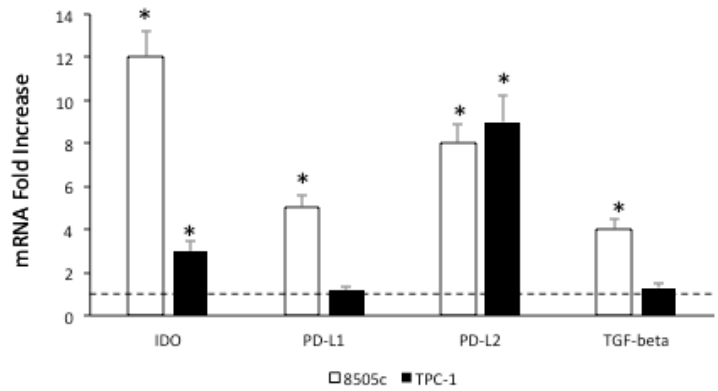

Figure 15: mRNA fold increase of the indicated immunomodulatory markers in 8505c OCT4/SOX2 or TPC-1 OCT4/SOX2 cells compared to empty vector transfected cells (dotted line). Mean ± SEM of three independent evaluations.*, $p<0.05$ compared to empty vector transfected cells.

Furthermore, we analysed the mRNA expression of the immune checkpoints PD-L1 and PD-L2, and of IDO in pBABE cells or in cells overexpressing OCT4/SOX2 grown in adherence and as thyrospheres. As shown in **Figure 16**, the expression levels of PD-L2 and IDO significantly increased in thyrospheres of 8505c pBABE cells compared to the same cells grown in adherence (dotted line). The expression of PD-L1 and IDO resulted significantly increased in 8505c OCT4/SOX2 thyrospheres in comparison to the same cells grown in adherence.

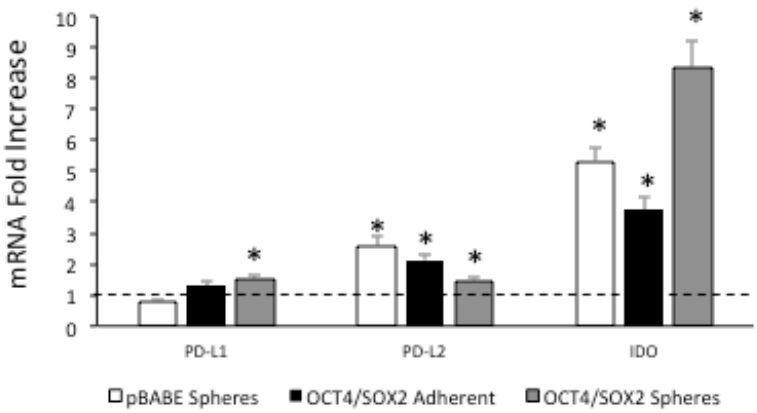

Figure 16: mRNA fold increase of IDO, PD-L1, and PD-L2 in 8505c pBABE thyrospheres, OCT4/SOX2 adherent or OCT4/SOX2 cl16 thyrospheres compared to the expression levels of 8505c pBABE cells grown in adherence (dotted line). Mean ± SEM of three independent evaluations. *, $p<0.05$ compared to 8505c pBABE adherent cells.

To confirm the above mentioned results also at the protein level we analysed the expression of PD-L1 and PD-L2 and of their receptor PD-1 in 8505c cells overexpressing or not OCT4/SOX2 by FACS analysis. We found that PD-L1 and PD-L2 protein levels increase in cells overexpressing OCT4 and SOX2 transcriptional factors **(Figure 17A, 17B)** with respect to control cells. Interestingly, TC cells displayed a detectable level also of the receptor PD-1. A further increase in its expression levels in particular in 8505c OCT4/SOX2 expressing cells was observed **(Figure 17)**. These observations support the hypothesis that stemness increases immunosuppressive features of TC cells. Since IL-8 increases stemness in a SLUG-dependent fashion, we verified, by similar approaches, whether PD-1, PD-L1 and PD-L2 expression could be regulated by IL-8 treatment. TC cells overexpressing the transcriptional factor SLUG or IL-8, that display increased stemness features, expressed higher levels of immunomodulatory molecules compared to control cells.

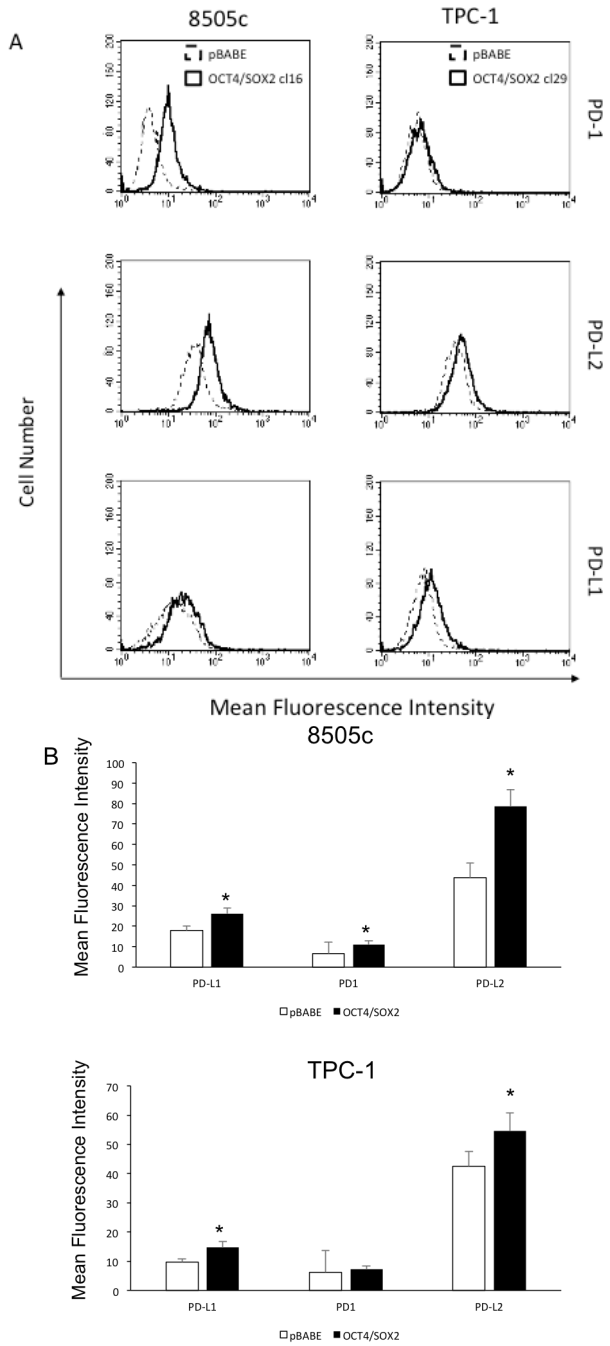

Figure 17: A) PD-1, PD-L1, and PD-L2 expression levels in 8505c or TPC-1 pBABE or one representative OCT4/SOX2 overexpressing clone ***B)****:* PD-1, PD-L1, and PD-L2 expression levels in 8505c or TPC-1 pBABE or OCT4/SOX2 overexpressing clones. Mean ± SEM of three experiments. *, $p<0.05$ compared to pBABE cells.

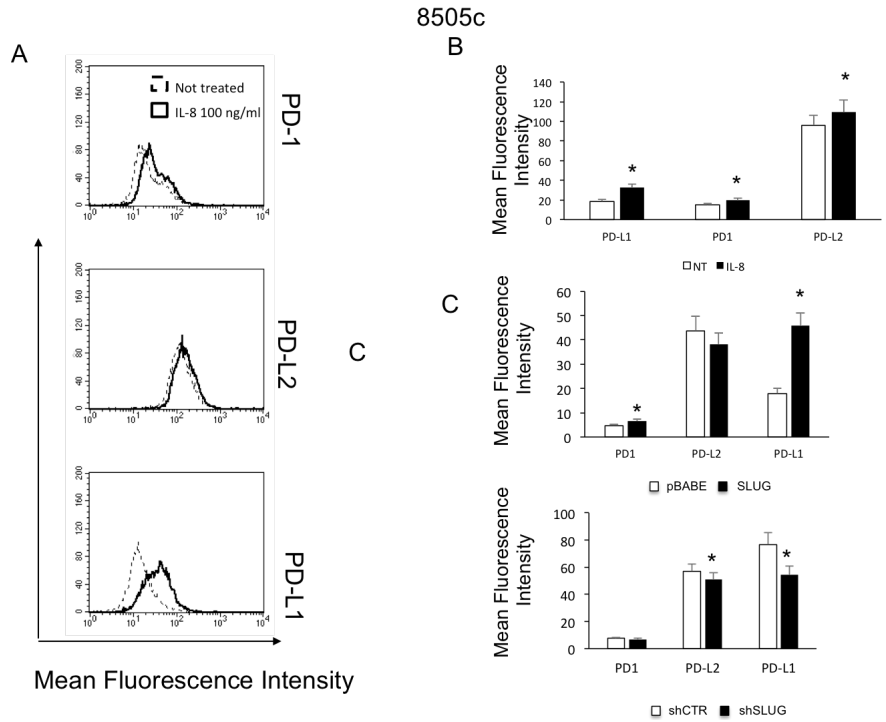

Figure 18: A) PD-1, PD-L1, and PD-L2 expression levels in 8505c parental cells treated or not with IL-8(100 ng/ml) for 24h. A representative experiment is shown ***B)*** PD-1, PD-L1, and PD-L2 expression levels in 8505c treated or not with IL-8 (100 ng/ml). Mean ± SEM of three experiments. *, $p<0.05$ compared to not-treated cells. ***C)*** PD-1, PD-L1, and PD-L2 expression levels in 8505c controls, overexpressing SLUG, or SLUG silenced (shSLUG) cells. Mean ± SEM of three clones. *, $p<0.05$ compared to controls.

By using both real-time PCR and FACS analyses we could confirm that IL-8 treatment causes an up-regulation of mRNA and protein levels of the immunomodulatory molecules **(Figure 18A, 18B)**. Consistently, 8505c SLUG cells, that exhibit increased EMT and stemness features [43], also featured increased PD-1 and PD-L1 protein levels compared to control cells. On the contrary, in 8505c cells silenced for SLUG, we observed a significant reduction of PD-L1 and PD-L2 protein levels with respect to control cells **(Figure 18C)**.

We also characterized in 8505c OCT4/SOX2 or 8505c SLUG cells the expression levels of IDO. We demonstrated that IDO protein levels and the percentage of IDO-positive cells increase in both 8505c and TPC-1 cell lines overexpressing OCT4/SOX2 compared to control cells **(Figure 19A)**. Consistently, SLUG overexpression in 8505c increased the expression of IDO, whereas its silencing was associated to a decrease in the expression of the IDO protein **(Figure 19B)**.

Taken together, these results suggest that stemness induction, either caused

by EMT-through IL-8 stimulation or SLUG enforced expression, or by direct OCT4/SOX2 enforced expression, correlates with the acquisition of an immunosuppressive phenotype of TC cells.

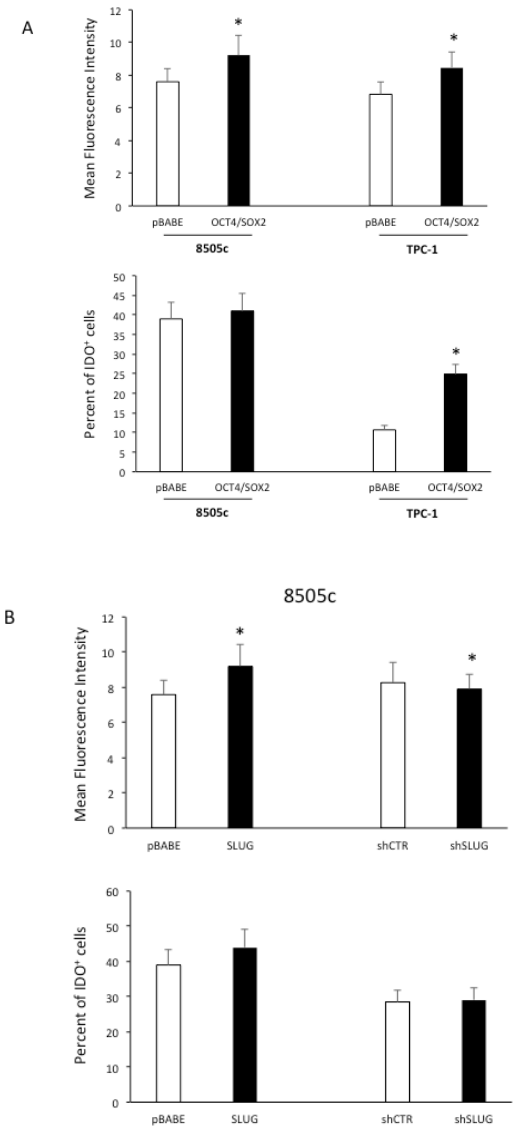

Figure 19: A) IDO expression levels and percentage of IDO positive cells in 8505c or TPC-1 transfected with the empty vector (pBABE) and in two clones overexpressing OCT4 and SOX2. Mean ± SEM of three clones. *, $p<0.05$ compared to empty vector transfected cells. ***19B)*** IDO expression levels and percentage of IDO positive cells in 8505c control, overexpressing SLUG, or SLUG silenced (shSLUG) cells. Mean ± SEM of three clones. *, $p<0.05$ compared to control cells.

To assess the expression of the immunomodulatory molecules in a panel of TC cells, we checked their expression levels in several PTC-derived (BcPAP, TPC-1) or ATC-derived (8505c, Cal62, SW1736, FRO, BHT101) cell lines compared to normal immortalized thyroid epithelial cells (Nthy-ori) (**Figure 20**). We found that the level of expression of both PD-L1 and IDO, but not PD-L2 (data not show), tend to increase in TC cell lines compared to control cells and it was in mean higher in ATC-derived compared to PTC-derived cell lines (**Figure 21**). Thus, immunosuppressive phenotype correlates with aggressiveness in TC cell lines.

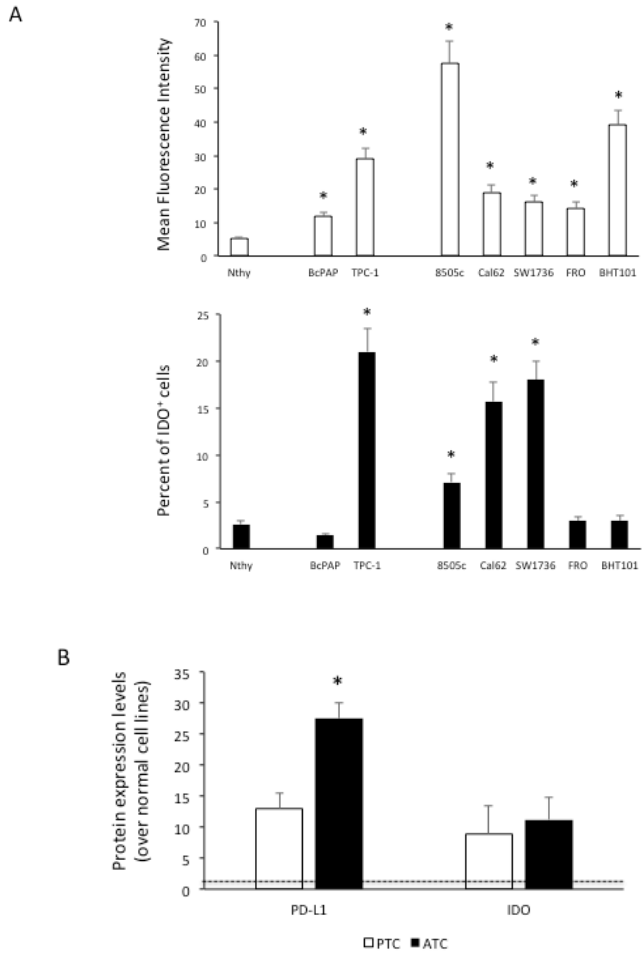

Figure 20: A*)** protein expression levels assessed by FACS analysis of PD-L1 and IDO in 2 PTC-derived and 5 ATC-derived cell lines compared the expression levels detected in Nthy-ori normal immortalized thyroid cells. Mean ± SEM of three determinations. $p<0.05$ compared to NThy cells ***B) Mean of ATC-derived compared to PTC-derived cell lines.

4.2.1 Functional role of immune checkpoints in TC

We then asked whether the up-regulation of the immunomodulatory molecules induced by OCT4 and SOX2 could sustain functional immunosuppression of T lymphocytes. To this aim, peripheral blood lymphocytes were isolated from different donors and tested for vitality both in the presence of TC cell-derived conditioned media (TC CM) or in a co-culture system with TC cell lines. We observed that TC cells overexpressing OCT4 and SOX2, when co-cultured with lymphoid cells, caused a significant reduction of lymphocyte vitality compared to control cells. These effects were not observed when lymphocytes were stimulated with conditioned media derived from the same cells (data not shown). Thus, OCT4 and SOX2 can increase the immunosuppressive functions of TC cells, possibly by the engagement of cell-cell contact mechanisms (i.e. PD-L1 and/or PD-L2) and not by the release of soluble factors.

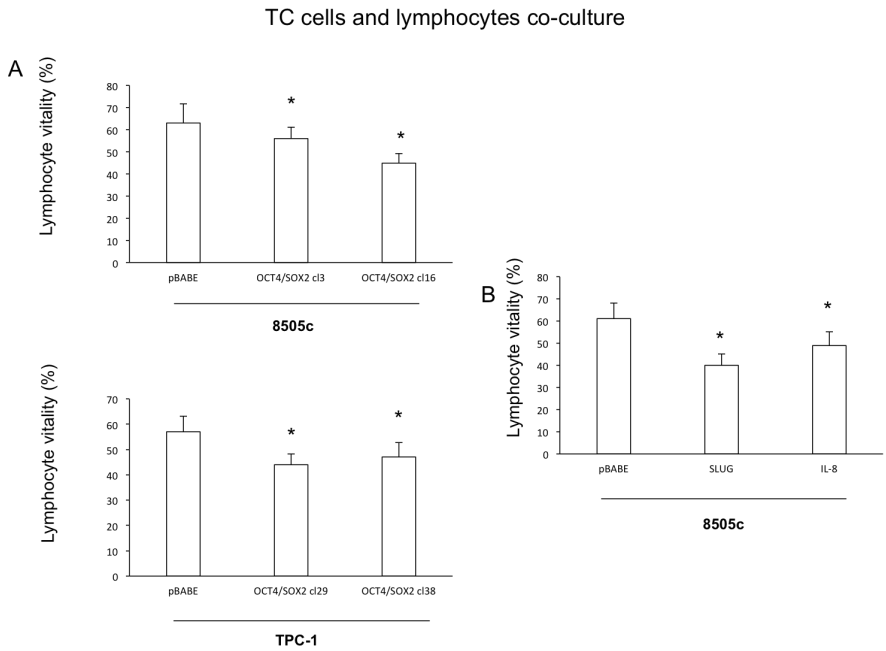

Figure 21: A) Percentage of vital T lymphocytes isolated from 3 different donors after 24 h of co-culture with 8505c or TPC-1 transfected with the empty vector (pBABE) or with two clones overexpressing OCT4 and SOX2. *, $p<0.05$ compared to empty vector transfected cells. ***B)*** Percentage of vital T lymphocytes isolated from 3 different donors after 24 h of co-culture with 8505c transfected with the empty vector (pBABE) and with an IL-8 or SLUG vector. The mean of three clones is shown. *, $p<0.05$ compared to empty vector transfected cells.

We then evaluated the expression levels of immunosuppressive factors in a model of TC in mice. To this aim, we used C57BL/6 mice expressing the

TRK-T1 oncogene under the control of the thyroid specific TG promoter [43]. TRK-T1 oncogene promotes the spontaneous development of tumours or hyperplastic lesions at 1 year (data not shown). At the same time point, we collected thyroids from both wild-type and transgenic mice, and analysed them for the expression of PD-1, PD-L1 and PD-L2 by Real-time PCR. We found a strong increase in the expression of PD-L1 in the pathological thyroids derived from the TRK-T1 mice with respect to the control thyroids **(Figure 22A)**. No increase in PD-1 or PD-L2 expression was observed. In line with this observation, the number of $CD8^+$ T lymphocytes infiltrating mouse thyroids was strongly reduced in TRK-T1 mice with respect to control **(Figure 22B)**. These data indicate that, similarly to what observed in other cancer types, also in thyroid carcinomas tumour progression is accompanied by the acquisition of immunosuppressive features.

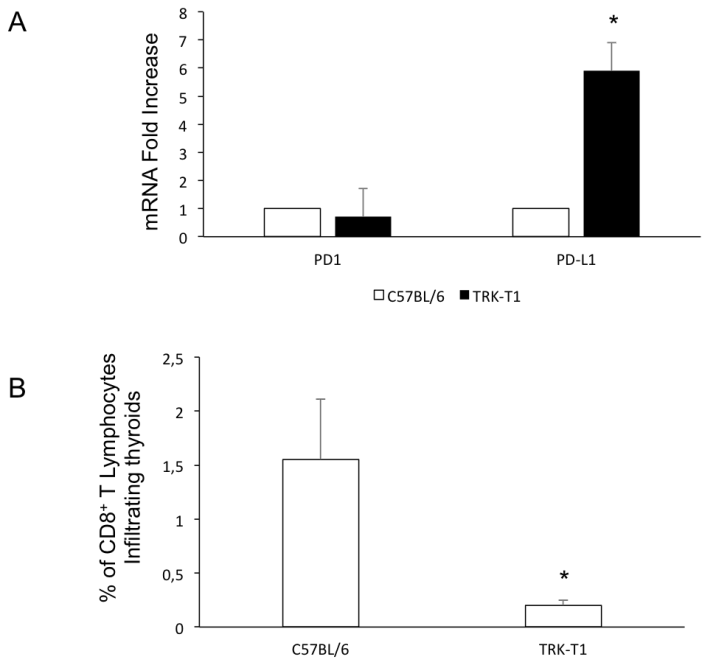

Figure 22: A) mRNA expression of PD-1 and PD-L1 in the thyroid tissues obtained from 3 control mice (C57BL/6) or 3 mice expressing TRK-T1 oncogene under the control of thyroid specific thyreoglobulin (TG) promoter. ***B)*** Percentage of $CD8^+$ T lymphocytes infiltrating mouse thyroids, as assessed by FACS analysis of the dissociated tissues obtained from 3 control mice (C57BL/6) or 3 mice expressing TRK-T1 oncogene, developing thyroid tumours. *, $p<0.05$ compared to controls

5. Conclusion/ Discussion

Thyroid cancer (TC) is the most common endocrine malignancy and its incidence is increasing. Despite well-differentiated forms, including papillary thyroid carcinomas (PTCs), follicular thyroid carcinomas (FTCs), have an excellent prognosis, with more than 90% cured by therapy, a small percentage (approx. 5%) of tumours becomes resistant to radioiodine therapy (radioactive iodine resistant-RAIR), the main treatment for these patients. Poorly differentiated thyroid carcinomas (PDTC), that invariably evolve in RAIR tumours, and anaplastic thyroid carcinomas (ATC), which are by definition RAIR, show limited options of treatment.

The scientific community believes that a small population of cells (cancer stem-like cells, CSCs) within the tumour is responsible for resistance to chemo-radiotherapy, recurrence and metastasis. Various studies supported this concept, thus strengthening the importance to characterize CSC populations in each cancer type. ATC, besides being the most aggressive among all thyroid cancers, and one of the most aggressive among all human cancer types, display stem-like features. ATCs features high levels of stemness markers and a stem-like behaviour. Recently, a CSC population has been identified in human ATCs samples, based upon the ability of this subpopulation to express high aldehyde dehydrogenase activity, to form thyrospheres in low adherence conditions and to form tumours in serial transplantation experiments. ATCs CSCs are more abundant and more aggressive than the CSC population found in PTCs [30]. In TC, stemness features correlate with the acquisition of Epithelial-to-Mesenchymal Transition (EMT) [78].

We have previously shown that the pro-inflammatory cytokine IL-8 induces stemness features in TC, through the induction of EMT [43]. We also found that IL-8 induces stemness properties in TC through the up-regulation of the SLUG EMT transcriptional factor [43]. Consistently, we found that IL-8 stimulation of TC cells induces the expression of stemness-related genes, including OCT4, SOX2, and NANOG. SOX2 and OCT4 stand at the top of a hierarchy governing the regulation of self-renewal, pluripotency and de-differentiation [79]. The roles of OCT4 and SOX2 have been described in various tumours [80].

Here, we generated OCT4 and SOX2-overexpressing PTC and ATC cell line models to characterize the effects of the two transcriptional factors in TC, and to obtain TC cells with enhanced stemness features, with which to study the features of TC stem-like cells. OCT4/ SOX2 TC cells show an increase in the proliferation rate and also in the death rate. These apparently contrasting results suggest that some of the genes activated in response to OCT4 and SOX2 may be responsible for an antiproliferative response such as apoptosis, senescence or other forms of cell-cycle arrest [74]. This phenomenon has been

defined as “reprogramming-induced senescence” [74]. Our data also support the finding that reprogramming factors can trigger apoptosis. It has been demonstrated for example that BAX is upregulated in response to the expression of OCT4, SOX2 and KLF4, and that the co-expression of the BAX antagonist BCL2 results in enhanced reprogramming efficiency [75]. It is possible that also in TC cells, the expression of the reprogramming factors OCT4 and SOX2 induces cell death by increasing the amount of pro-apoptotic proteins. We are currently analysing this possibility.

In both ATC (8505c) and PTC (TPC-1) cells, enforced expression of OCT4/SOX2 caused an increase in cell motility, as assessed by migration assays. Moreover, OCT4/SOX2 induced an enhancement of stemness features, including self-renewal and tumorigenic potential, as assessed by self-renewal and limiting dilution assays and tumorigenicity experiments in immunodeficient mice.

There is increasing evidence that CSCs are responsible for the aggressive behaviour of tumours. This is not only due to CSC resistance to anti-cancer therapy and increased metastatic activity, but also to the ability of CSCs to evade the immune system. CSCs possess features similar to normal stem cells in their ability of inducing immune modulation. Unfortunately, possession of these features by CSCs contributes to their escape from the immune system recognition.

Here, we demonstrated that stemness is closely linked with immunosuppressive phenotype in TC cells. In fact, TC cells with increasing aggressive features also display increased immunsoppressive functions. The immunosuppressive phenotype of TC cells is characterized by increased expression of PD-L1, PD-L2, IDO, observed in PTCs versus normal thyroid cells, and in ATCs versus PTCs. Moreover, OCT4/SOX2 overexpressing TC cells display increased immunosuppressive features in comparison to parental cells. Not only were immunosuppressive molecules increased in OCT4/SOX2 TC cells, but also immunosuppressive activity on lymphocytes was enhanced. In fact, lymphocyte vitality was significantly reduced when co-cultured with OCT4/SOX2 TC cells when compared to control cells. This effect was not observed when lymphocytes were treated with conditioned media form TC cultures, but only when lymphocytes were co-cultured with TC cells. These observations indicate that OCT4 and SOX2 can increase the immunomodulatory functions of TC cells, possibly by the engagement of cell-cell contact mechanisms (i.e. PD-L1 and/or PD-L2). In support of these findings, we analysed the behaviour of immunosuppressive molecules in a mouse model of TC, in which the TRK-T1 oncogene is placed under the transcriptional control of the thyroid-specific TG gene promoter, thus causing the occurrence of thyroid hyperplasia and thyroid tumours. By comparing the immunosuppressive features of normal and pathological thyroids of these mice, we found an increase of the expression of PD-L1 in

the thyroids derived from the TRK-T1 mice with respect the control thyroids. Importantly, the increase in PD-L1 correlated with a decrease of $CD8^+$ T lymphocytes infiltrating TG TRK-T1 thyroids. Therefore, TC display immunosuppressive features, and these features correlate with TC aggressiveness; moreover stemness features could enhance immunosuppressive ability of TC.

A limited number of reports indicate that the immunomodulatory properties of CSCs derived from several tissues could be linked specifically to SOX2 and/or OCT4 expression. Zhong *et al*. found that both SOX2 and PD-L1 were expressed at a markedly higher level in hepatocellular carcinoma (HCC) tissues in comparison to adjacent non-tumour tissues [81]. Moreover, the expression levels of both genes were correlated with each other. Knockdown of SOX2 reduced the cell proliferation ability and induced apoptosis of HCC cells [81]. Noteworthy, the depletion of SOX2 also reduced the expression of PD-L1. Further analysis showed that there is a consensus SOX2 binding site in the promoter region of PD-L1. It has been also demonstrated that SOX2 directly bound to the PD-L1 promoter through the consensus SOX2 motif [81]. Further evidence by luciferase reporter assays revealed that SOX2 promoted the transcription activity of PD-L1 promoter region through the SOX2 motif. Collectively, these data provide a novel insight into the function and the interplay of SOX2 and PD-L1 in HCC.

Moreover, there are also evidence that PD-L1 can regulate stemness. In fact, specific knockdown of PD-L1 using shRNA revealed its critical role in the expression of OCT4 and NANOG in breast CSCs [82]. Conversely, these factors could be induced upon PD-L1 ectopic expression in cells that are normally PD-L1 negative. Most importantly, down-regulation of PD-L1 compromised the self-renewal capability of breast CSCs *in vitro* and *in vivo* as shown by tumoursphere formation assay and extreme limiting dilution assay, respectively. This study demonstrates a novel role for PD-L1 in identifying and sustaining CSCs of breast [82].

There is growing interest toward therapies that target the CSC population within tumours. Moreover, immunotherapies directed against the PD-1/PD-L1/PD-L2 circuit raised great expectations in the cure of various cancer types given the successful results obtained in the first clinical trials. Our data indicate that targeting stemness pathways in TC might not only inhibit its malignant features, but also limit its immunosuppressive capacity.

LIST OF PUBLICATIONS

Liotti F, Collina F, Pone E, La Sala L, Franco R, Prevete N, Melillo RM. "*Interleukin-8, but not the Related Chemokine CXCL1, Sustains an Autocrine Circuit Necessary for the Properties and Functions of Thyroid Cancer Stem Cells"*
Stem Cells. 2016 Aug 31. [Epub ahead of print]. Online *ISSN: 1549-4918*

ACKNOWLEDGMENTS

I carried out my study in the Molecular Medicine and Medical Biotechnology Department of Federico II University of Naples.
Foremost, I would like to express my sincere gratitude to my tutor Prof. Rosa Marina Melillo for the continuous support of my PhD study and research, for her patience, and immense knowledge. Her guidance helped me in all the time of research and writing of this thesis. I want to thank Dott. Liotti Federica and Dott. Prevete Nella for their active participation and support in my PhD project for helping me in the experiments and stimulated discussion.
I also want to thank all people that I meet during my PhD experience.
I also want to thank Francesco for his love.
Last but not the least my gratitude goes to my family for their encouragement and faith in me.

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