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ESTROGENS AND THYROID CANCER IS A STEM AFFAIR. A PRELIMINARY STUDY

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Chapter 1

Estrogens and Stem Cells in Thyroid Cancer

Abstract

Recent discoveries highlight the emerging role of estrogens in the initiation and progression of different malignancies through their interaction with stem cell compartment. Estrogens play a relevant role especially for those tumors bearing a gender disparity in incidence and aggressiveness, as occurs for most thyroid diseases. Although several experimental lines suggest that estrogens promote thyroid cell proliferation and invasion, their precise contribution in stem cell compartment still remains unclear. This review underlines the interplay between hormones and thyroid function, which could help to complete the puzzle of gender discrepancy in thyroid malignancies. Defining the association between estrogen receptors' status and signaling pathways by which estrogens exert their effects on thyroid cells is a potential tool that provides important insights in pathogenetic mechanisms of thyroid tumors.

Introduction

The endocrine system consists of a network of glands secreting hormones, which are chemical messengers that cooperate in growth, development, metabolism, and reproductive functions. The largest endocrine organ in the human body is the thyroid gland, whose function is the systemic metabolic regulation through **Thyroid Hormones (THs)** produced by follicular cells, and Calcitonin produced by parafollicular cells. Different malignancy histotypes can arise from these cells: Papillary (PTC), Follicular (FTC), and Anaplastic Thyroid Carcinomas (ATC) originate from follicular cells, while Medullary Thyroid Carcinomas (MTC) derive from parafollicular cells (Kondo, Ezzat et al. 2006). Notably, more than 95% of Thyroid Carcinomas (TCs) arise from follicular cells. These malignancies are indolent tumors treated by surgical resection with or without radioactive-Iodine ablation since they maintain their distinct potential to concentrate Iodine. The loss of typical thyroid cell characteristics and functions, including expression of the **Thyroid-Stimulating Hormone (TSH)** receptor (TSH-R), Thyroglobulin (Tg), Thyroid Peroxidase (TPO), and Sodium Iodide Symporter (NIS), defines the hallmark of ATCs, which are lethal malignancies with no effective therapy (Kondo, Ezzat et al. 2006; Nikiforova and Nikiforov 2008; Smallridge, Marlow et al. 2009).

Besides genetic alterations in **Mitogen-Activated Protein Kinase** (**MAPK**), **PI-3 kinase** (**PI3K**), and TSH signaling pathways, thyroid carcinogenesis is fostered by the microenvironment, Growth Factors (GFs), and various hormones, including estrogens (Rahbari, Zhang et al. 2010). Hormones can set off a cascade of signaling pathways, enhancing or contrasting specific effects triggered by other factors. Based on this scenario, the role of estrogens has been proposed in the pathogenesis of thyroid proliferative and neoplastic disorders. This hypothesis is supported by data regarding gender incidence, which reported a frequency of thyroid nodules about three to four times higher in women than in men with a peak rate occurring earlier in women (Rajoria, Suriano et al. 2012; Siegel, Naishadham et al. 2013). Furthermore, the clarification of the estrogen-driven pathogenesis could be crucial in explaining why PTC constitutes the seventh most common cancer in the female gender (Rajoria, Suriano et al. 2010; Xu, Chen et al. 2013). An in vivo study reported that circulating estrogens are directly responsible for the increased female susceptibility to thyroid disease, through PI3K pathway activation and repressing p27 expression. The authors also observed a significant estrogen role in the transcriptional regulation of TPO, DUOX1, and NIS genes (Antico-Arciuch, Dima et al. 2010). Although several studies have demonstrated a direct action by estrogens on thyroid growth and function (Zeng, Chen et al. 2007; Ceresini, Milli et al. 2008; Rajoria, Suriano et al. 2010; Vaiman, Olevson et al. 2010), the precise mechanism underlying the proliferative and neoplastic disorders still remains undefined. In particular, it would be interesting to explore the role of hormones in TC initiation.

The cellular origin of thyroid carcinomas has been explained by different models (FIG. 1). The **multistep carcinogenesis model** predicts that TC originates from follicular cells as a consequence of multiple mutations accumulated throughout their life-span. These events are characterized by a dedifferentiation process with a marked Epithelial-to-

Mesenchymal Transition (EMT), in which well-differentiated TC cells transform into a more undifferentiated phenotype (Kondo, Ezzat et al. 2006). The **fetal cell carcinogenesis model** hypothesizes that TC cells would be generated by transforming three types of fetal thyroid cells, Stem Cells (SCs), thyroblasts, and prothyrocytes, which result in ATC, PTC, and FTC, respectively (Takano and Amino 2005; Takano 2007). The heterogeneity of tumor bulk had led to a **Cancer Stem Cells (CSCs) model** to propose TC as a SC disease. The growing body of experimental evidence has revealed that an accumulation of genetic abnormalities in tissue-resident SCs or in their more committed progenies, concomitant with the niche epigenetic alterations, result in their malignant transformation (Vermeulen, Sprick et al. 2008; Lin 2011).

The "cell-of-origin" concept explains how a normal cell acquires the first alteration able to trigger tumor initiation (Tumor-Initiating Cells, TICs) (Visvader 2011). Wnt pathway plays a crucial role in SC/progenitor compartment maintenance, and has been described in several tumors, including TC, resulting in nuclear β-catenin-induced proliferation (Todaro, Iovino et al. 2010; Sastre-Perona and Santisteban 2012; Van Camp, Beckers et al. 2014).

In this review, the most current findings supporting the carcinogenesis effects of estrogens and THs, will be addressed. A special emphasis will be given to the role of exogenous and endogenous GFs affecting thyroid proliferative pathways in SC compartment.

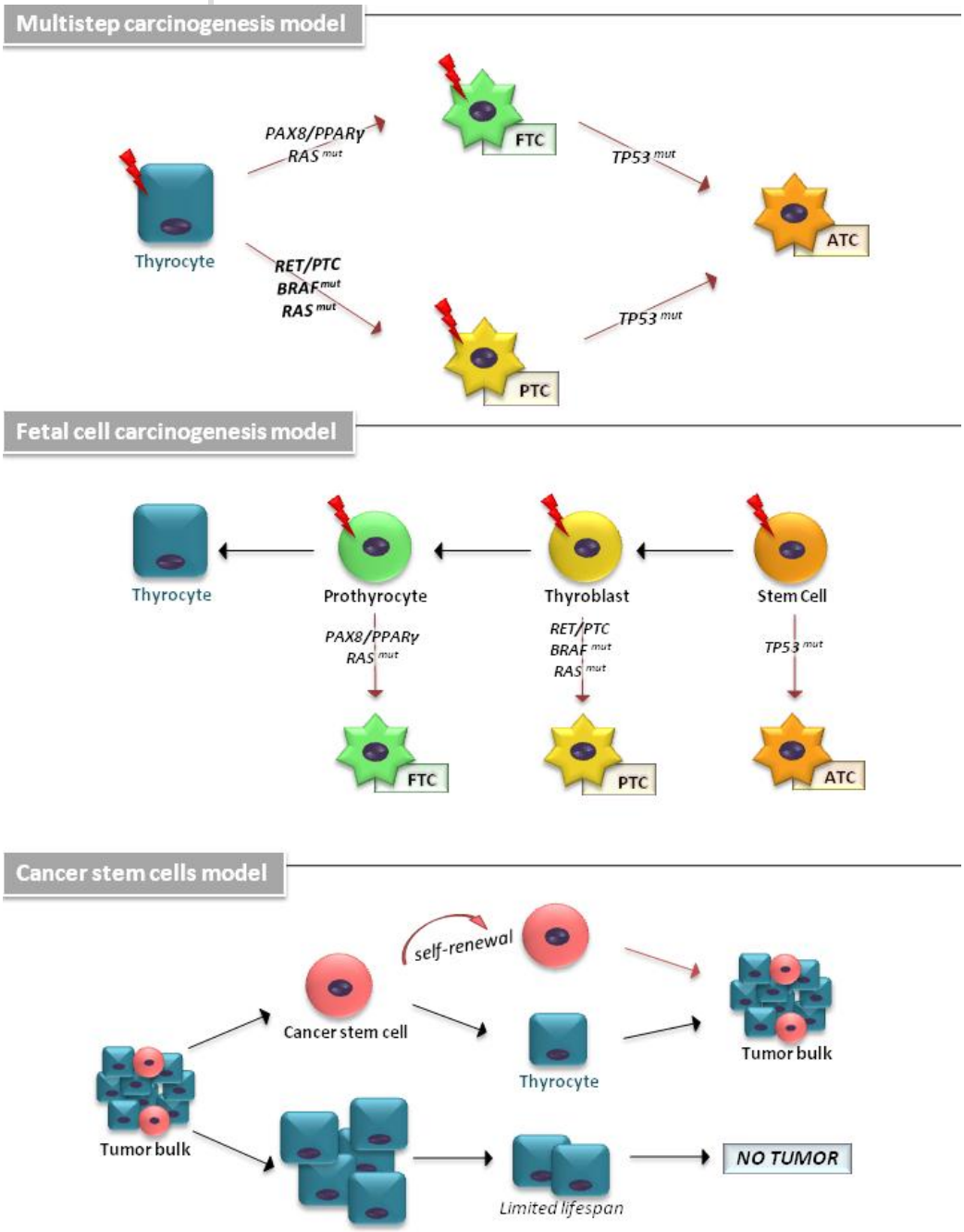

Figure 1. The cellular origin of thyroid carcinomas. According to the **multistep carcinogenesis model,** TC originates from follicular cells as a consequence of multiple mutations accumulated throughout their life-span. Thyrocytes could give rise to PTC by RAS and BRAF mutations or RET/ PTC and NTRK1 rearrangements and to FTC by point mutations of the RAS gene and PAX8/ PPARγ rearrangement. ATC derive from PTC and FTC after deregulation of the p53 and the Wnt/β-catenin pathway.
In **fetal cell carcinogenesis model,** three types of fetal thyroid cell were proposed to generate different forms of thyroid cancer. Fetal thyroid stem cells, characterized by expression of the oncofetal fibronectin (OF), generate ATC, thyroblasts, which express OF and the differentiation marker Tg, are proposed to be the cellular origin of PTC. The more differentiated prothyrocytes, expressing Tg, give rise to FTC.
The **Cancer Stem Cells model** proposes TC as a SC disease. The accumulation of mutations in differentiated thyrocytes leads to their transformation. A subset of these cells may (in more aggressive tumor types) dedifferentiate and assume CSC characteristics.

Estrogens

As recently published by Morrison's research group, **estrogens** are involved in increasing hematopoietic SC self-renewal in females subjects and more specifically during pregnancy (Nakada, Oguro et al. 2014). It is likely that normal and tumor thyroid tissues, which express Estrogen Receptors (ER), could be subject to the same mechanism of estrogen action (Manole, Schildknecht et al. 2001; Lee, Chen et al. 2005; Zeng, Chen et al. 2007; Dong, Zhang et al. 2013).

Involved in cellular processes such as growth, cell motility, and apoptosis, in reproductive tissues and other organs, including endocrine glands, estrogens are mainly produced by the adrenal cortex and ovary, but also by the thyroid (Santin and Furlanetto 2011; Antico Arciuch and Di Cristofano 2012). They are present in women and men with a notable increase in women at reproductive age. The three principal estrogens, **Estrone (E1)**, **Estradiol (E2)**, and **Estriol (E3)**, are processed in metabolites with different estrogenic abilities, which create a different risk in developing cancer (Thiruvengadam, Govindarajulu et al. 2003; Heldring, Pike et al. 2007; Zahid, Goldner et al. 2013).

E2 is the most potent estrogen since it has the highest affinity to its receptors. Estrogens perform their function by binding to **ER alpha and beta (ER-α, ER-β)**, and a transmembrane intracellular non-classical ER **G protein-coupled receptor 30 (GPR30)** (FIG. 2). ER-α and ER-β are soluble intracellular nuclear receptors, belonging to a ligand-dependent nuclear receptor superfamily of Transcription Factors (TFs) (Santin and Furlanetto 2011; Antico Arciuch and Di Cristofano 2012). ER-α is the key factor of E2-induced proliferation with an anti-apoptosis effect. In females of reproductive age, ER-α levels are higher in PTC compared to nodular goiter patients, showing a positive correlation between ER-α and Ki-67 expression levels. In contrast, ER-β is associated with apoptosis and growth inhibition, providing a negative correlation with mutant P53 (Huang, Dong et al. 2014). PPARγ also interacts with ER-α inhibiting each other, and with ER-β enhancing their inhibitory effect on cell proliferation and migration (Chu, van Hasselt et al. 2014). In light of this, the ER-α/ER-β *ratio* could be helpful to elucidate the TC pathophysiology (Leitman, Paruthiyil et al. 2010; Santin and Furlanetto 2011).

The interaction between estrogens and ERs signals through different pathways:

- **Genomic (or classical) estrogen-signaling**: after accessing the cell through passive diffusion, E2 binds to ER, which changes its conformation and homo- or heterodimerizes (E2-ER). This complex translocates into the nucleus, where it binds to the 15-bp palindromic Estrogen Response Element (ERE) located in the regulatory regions of target genes. This interaction leads to a co-activators recruitment, which in turn allows expression of genes involved in proliferation (Klinge 2000; Nilsson, Makela et al. 2001).

- **ERE-independent genomic actions (TFs cross-talk)**: ERE-lacking genes can be activated by modulating other TFs through protein-protein interactions. This molecular mechanism induces chromatin remodeling, histone unwinding, and

interaction with the basal transcription machinery complex (Bjornstrom and Sjoberg 2005; Ascenzi, Bocedi et al. 2006; Osmanbeyoglu, Lu et al. 2013).

- **Non-genomic (or membrane-initiated) estrogen-signaling**: E2 activation of plasma membrane-associated ER and GPR30 promotes the MAPK and PI3K signaling pathways and/or increases the Ca^{2+} levels (Acconcia and Kumar 2006; Zeng, Chen et al. 2007; Chen, Brown et al. 2008; He, Cai et al. 2009). They can also activate G proteins resulting in cAMP production, similar to TSH-signaling in thyrocytes, and assist the activation of metalloproteinases (MMPs) and the GF pathway (Rajoria, Suriano et al. 2012).

- **Ligand-independent signaling**: in absence of E2, GFs can stimulate ERs directly or indirectly through MAPK and/or PI3K pathways (Roman-Blas, Castaneda et al. 2009).

The cross-talk between genomic and non-genomic pathways, as well as the integrative signaling by E2 in different cell compartments, leads to a synergy that provides plasticity in cell response. Estrogens dispatch their proliferative role also by increasing T_3 levels and stimulating the Iodine-uptake and TPO activity (Lima, Barros et al. 2006).

Furlanetto and coworkers reported that E2 increases proliferation of thyroid cells down-regulating NIS (Furlanetto, Nguyen et al. 1999). These data underlines the pivotal role of estrogens in the SC compartment maintenance. In normal and tumor thyroid cell lines, Rajoria *et al*. documented that E2 is associated with increased proliferation, adhesion, invasion, and migration *via* β-catenin (Rajoria, Suriano et al. 2010) and MMP-9 modulation (Rajoria, Suriano et al. 2011). Likewise, E-cadherin down-regulation and β-catenin translocation sustain the metastatic activity of TC cells (Dong, Zhang et al. 2013).These results confirmed the findings by Kouzmenko *et al.*, which reported the first evidence of cross-talk between estrogens and Wnt pathways through functional interaction of β-catenin with ER-α (Kouzmenko, Takeyama et al. 2004).

Xu *et al.* analyzed whether differentiated and SC/progenitors could be target of estrogen action in thyroid (Xu, Chen et al. 2013). SCs isolated from goiter tissue enhanced their sphere-forming ability in presence of E2. Moreover, thyroid-sphere cells showed ER-α mRNA levels eight times higher than those of more differentiated thyrocytes. This suggests the gender discrepancy in TC incidence, and a difference in terms of aggressiveness and survival.

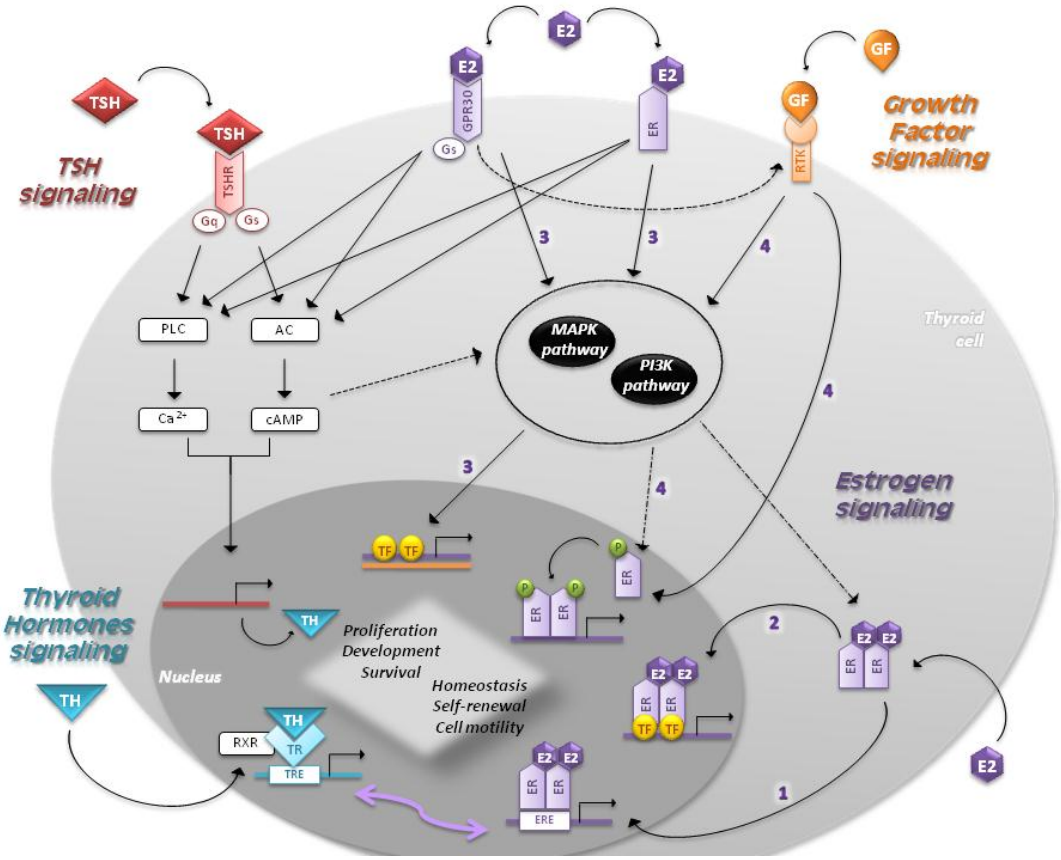

Figure 2. Signaling pathways in follicular cells. The main regulators of thyroid proliferation and function act through TSH signaling and GF pathway. THs control the secretion of TSH, which binds to TSH-R and induces the coupling of G-proteins stimulating AC and PLC. TSH also acts *via* PI3K pathway. GFs act *via* MAPK and PI3K pathways regulating the expression of genes involved in survival, cell cycle progression, and proliferation.

Estrogens regulate proliferation, cell motility, differentiation, and apoptosis through four different mechanisms: 1) *Genomic (or classical) estrogen-signaling*: E2-ER complex translocates into the nucleus, where it binds to ERE-sequences; 2) *ERE-independent genomic actions (TFs cross-talk)*: genes lacking in ERE-sequences are activated by other TFs in the nucleus through protein-protein interactions; 3) *Non-genomic (or membrane-initiated) estrogen-signaling*: E2 activation of plasma membrane-associated ER and GPR30 trigger the activation of MAPK and PI3K pathways and/or increases the Ca^{2+} levels; 4) *Ligand-independent signaling*: in absence of E2, GFs can stimulate ERs directly or indirectly through MAPK and/or PI3K pathways.

THs play a critical role in development and homeostasis. Nuclear TRs activate gene expression by binding to RXR which in turn bind to TRE-sequences. Given that EREs share a similar nucleotide sequence with TREs, ERs and TRs can interact and regulate several transcriptional responses. The cross-talk between genomic and non-genomic pathways and other integrative signaling leads to a synergic cell response.

Thyroid Hormones

THs control the secretion of **Thyrotropin-Releasing Hormone (TRH)** from the hypothalamus and **TSH** from the anterior pituitary through negative feedback loops (Kondo, Ezzat et al. 2006). Thyroid homeostasis and function are regulated by a concert of signals accumulated from TSH and GF pathways. TSH binds to TSH-R and induces the coupling of different G-proteins, stimulating Adenylate Cyclase (AC) and Phospholipase C (PLC) (FIG. 2). This promotes Iodide uptake and TG, TPO, and NIS expression, producing Thyroxine (T_4) and Triiodothyronine (T_3) (Postiglione, Parlato et al. 2002; Sastre-Perona and Santisteban 2012). On the contrary, intracellular Ca^{2+} and PLC regulate Iodine release, H_2O_2 production, and Tg iodination (Medina and Santisteban 2000; Kimura, Van Keymeulen et al. 2001). Although cAMP is the main mediator of TSH stimulation in thyroid cell growth, TSH *via* PI3K increases cyclin E levels leading to cell cycle progression (Cass, Summers et al. 1999; Roger, van Staveren et al. 2010). TSH-R is also associated to the MAPK pathway through its desensitization and internalization apparatus (Kursawe and Paschke 2007).

Gain-of-function mutations in TSHR or Gs genes result in increased cAMP accumulation and TSH-independent proliferation, which account for hyperfunctioning nodules in patients with multinodular goiters (Tallini 2002; Krohn, Fuhrer et al. 2005). These alterations result insufficient for the malignant transformation of thyroid cells (Matsuo, Friedman et al. 1993; Spambalg, Sharifi et al. 1996). Hence, it is likely that other factors intervene in the SC compartment, which is assumed to be the target of neoplastic transformation. Alterations of the Wnt pathway effectors are involved in cancer initiation and progression (Reya and Clevers 2005). In particular, TSH-mediated Wnt-1 over-expression and GSK-3β inhibition promote thyroid cell proliferation (Kim, Lewis et al. 2007; Chen, Jiang et al. 2010).

THs play a critical role in the tissue development and homeostasis by direct transcriptional regulation or modulation of different pathways (Pascual and Aranda 2013). Although T_4 is the predominant hormone produced by the thyroid, T_3 is the active form that mediates gene regulation binding with a higher affinity to **Thyroid Receptors (TRs)** (Cheng, Leonard et al. 2010). Nuclear TRs activate gene expression by binding with the Retinoid X Receptors (RXRs) to TH Response Elements (TRE), located on the promoters of target genes (FIG. 2) (Yen, Ando et al. 2006). Given that EREs share a similar nucleotide sequence with TREs, ERs and TRs can interact and regulate several transcriptional responses to environmental *stimuli* (Rajoria, Suriano et al. 2012). Interestingly, ERE can act as a Peroxisome Proliferator Responsive Elements (PPRE), binding PPARγ/RXR. It can henceforth inhibit ER transactivaction through a competition for ERE binding (Keller, Givel et al. 1995). In line with this cross-interaction, the proliferative effect of estrogens on human NPA-87-1 PTC cell line is TSH-independent (Banu, Govindarajulu et al. 2002). Lima *et al.* demonstrated a more direct proliferative effect since E2 administration to prepubertal and adult rats enhances thyroid weight without significant changes in T_3, T_4, and TSH hematopoietic levels (Lima, Barros et al. 2006).

Recent studies in human cancers and mouse models provide strong evidence that the loss of TRs function contributes to cancer initiation and progression (Kim and Cheng 2013). While the TRα1 trigger directly promotes transcription of CTNNB1 (Plateroti, Kress et al. 2006; Kress, Samarut et al. 2009), the effect generated by the TRα2 stimulation in SC compartment is still unknown. Cross-talk between THs-TRα1 and Wnt pathway has been confirmed by the up-regulation of several SC markers (Kress, Skah et al. 2010). Furthermore, it was reported that aberrant nuclear localization of β-catenin induced by CTNNB1 mutations contributes to the progression of ATCs (Garcia-Rostan, Camp et al. 2001). Data reported by Todaro *et al.* showed that E-cadherin down-regulation together with β-catenin activation, confer an invasive capacity and higher metastatic rate to thyroid CSCs (Todaro, Iovino et al. 2010).

Growth Factors

In thyroid, **GFs** exert their proliferative effects by inducing the RTK dimerization that activates the downstream PI3K pathway and the MAPK cascade *via* G proteins (FIG. 2). Alterations in genes involved in the MAPK pathway led to its constitutive activation, which represents a typical feature of TC (Kondo, Ezzat et al. 2006). In particular, mutations in RET and NTRK and alterations in RAS and BRAF intracellular signal-transducers are clearly implicated in PTC pathogenesis (Nikiforov and Nikiforova 2011). RAS point mutations and PAX8/PPARγ rearrangement have been frequently implicated in FTC pathogenesis (Kroll, Sarraf et al. 2000; Garcia-Rostan, Zhao et al. 2003). The inactivation of RASAL1 (encoding a RAS GTPase-activating protein) by hypermethylation and mutations provides a new genetic background for FTCs and ATCs (Liu, Yang et al. 2013). Besides nuclear β-catenin accumulation and p53 inactivation, oncogenic activation of MAPK and PI3K/Akt/Fox03a are frequently found in ATC (Nikiforova and Nikiforov 2008; Bellelli, Castellone et al. 2012; Nehs, Nucera et al. 2012). The acquisition of a TERT promoter mutation was recently associated with clinic-pathological aggressiveness in FTCs and BRAF mutation-positive PTCs (Landa, Ganly et al. 2013; Liu, Yang et al. 2013).

The mesenchymal tissue is involved in thyroid development being that it releases **Pro-epidermal growth factor (EGF)** and **Basic fibroblast growth factor** (**FGF-2**), promoting cell proliferation and repressing differentiation (De Felice and Di Lauro 2004; Fagman and Nilsson 2011). Estrogens play a pivotal role in this context by inducing the production of EGF and other TFs, such as TGF-α (Rajoria, Suriano et al. 2012).

After **EGF** binding, RTKs of the ErbB family (EGFR/ErbB1, ErbB2, ErbB3, and ErbB4) achieve activation through the arrangement in homo- and/or heterodimeric complexes (Yarden and Sliwkowski 2001; Konturek 2012). In thyroid, TSH increases the expression of EGFRs that in turn promote the EGF mitogenic effect and contribute to gland homeostasis. The combination of specific EGFRs regulates the stimulation intensity, inducing transformation. Indeed, an increased expression of EGFRs in TCs compared to normal tissue has been reported (Kato, Kobayashi et al. 2004). EGFR/ErbB1 over-expression and its constitutive phosphorylation have been observed on ATC samples

and cell lines (Bergstrom, Westermark et al. 2000). Their expression has been retrieved in 90% of the PTC samples examined by Song (Song 1991). In combination with the repression of VEGF, EGF inhibitors could be a promising therapy for ATCs as demonstrated by *in vitro* studies (Schiff, McMurphy et al. 2004; Hoffmann, Glaser et al. 2006). EGF is also supplemented in the serum-free culture medium used to isolate SCs and CSCs *in vitro* (Lan, Cui et al. 2007; Todaro, Iovino et al. 2010; Zheng, Cui et al. 2010; Malaguarnera, Frasca et al. 2011; Tseng, Huang et al. 2012; Li, Reeb et al. 2013; Ahn, Henderson et al. 2014)

Similarly, the cell response to **FGF** is regulated by FGF RTKs (FGFRs 1-4). FGF-2 exerts autocrine and paracrine stimulatory effects on thyroid growth, since the basement membrane of thyrocytes is able to produce FGF itself. FGF is also used *in vitro* for the maintenance of SC niche (Todaro, Iovino et al. 2010; Longmire, Ikonomou et al. 2012); in particular, it could have an inhibitory effect on thyroid function through cAMP inhibition and TSH's activity weakening (Konturek 2012). In TC, increased FGF-2 levels and FGFR2 over-expression are critical in tumor progression and neovascularization (Eggo, Hopkins et al. 1995; Guo, Liu et al. 2012). Therefore, the differential expression in normal and malignant conditions could make this receptor a potential diagnostic marker for TCs (Redler, Di Rocco et al. 2013).

GFs also affect development and metabolic processes through **Insulin-Like Growth Factor (IGF)**. After binding of their ligands, IGF receptors (IGF-Rs) autophosphorylate their intracellular domain and activate the MAPK and PI3K cascade (Laron 2001). Consistently, IGF enhances the TSH mitogenic effect on follicular cells (Bendall, Stewart et al. 2007); on the other hand, it also cooperates with FGF-2 in establishing and maintaining the SC niche *in vitro* (Bendall, Stewart et al. 2007). Indeed, IGF pathway effectors are over-expressed in CSCs: IGFR2 is involved in an autocrine loop that sustains SC renewal, and IGF increases the expression of Oct-4 and Nanog when added to the culture medium (Vella, Pandini et al. 2002; Malaguarnera, Frasca et al. 2011; Malaguarnera and Belfiore 2014).

Estrogen-Growth Factors interacting proteins

Recently, there has been a focus on importance of the ER-GFs interacting proteins on cancer cell proliferation and invasivity. An example is **Mediator of ERbB2-driven cell motility (MEMO),** which enhances ER-α extra-nuclear functions through the interaction with IGFR1 and ERbB2, activating MAPK and PI3K signaling (Jiang, Yang et al. 2013).

Concluding Remarks

Since the theory of fetal carcinogenesis has initially been postulated, thyroid CSCs have been studied for their potential role as TICs. It has been hypothesized that various factors could be involved in the malignant transformation, such as aberrant molecular events converging to RTK, MAPK and PI3K pathway activation. Besides the oncogenes contribution, it is likely that a network of various hormones and GFs could maintain the SC niche and enhance the proliferation of progenitors sustaining tumor bulk growth. Indeed, recent studies demonstrate that sexual hormones could exert a supportive role in the propagation of SCs and progenitors, as suggested by the cross-talk between estrogen signaling and Wnt pathway. Furthermore, the latter pathway has also been observed interacting with THs in SC compartment and so accelerating tumorigenic processes. This mechanism could be benefited by the interaction between different cascades, which enhances or contrasts specific cellular response in tumor conditions. In conclusion, an in depth study on the concert between estrogens, THs, and GFs could be helpful to elucidate hormones-driven thyroid carcinogenesis. Gaining more insight into this interaction could also explain the gender imbalance in tumor incidence for the purpose of identifying a more targeted approach in TC therapy.

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> Estrogens and Thyroid Cancer is a stem affair. A preliminary study

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Chapter 2

Estrogen and Thyroid Cancer is a stem affair. A preliminary study

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Abstract

In the thyroid gland, nodules occur with a prevalence three to four times higher in females than in males. Papillary Thyroid Cancer has an incidence of 3:1 when comparing women to men with different aggressiveness. This gender discrepancy suggests a specific function of sexual hormones that could interact with thyroid function enhancing hyperproliferative and/or malignant conditions. It has been reported that estrogens are involved in increasing stem cell self-renewal, suggesting a role in the tumor trigger. This study aims to contribute to the understanding of the pathogenesis and the underlying causes of gender differences in thyroid cancer. This will be obtained by studying the specific role of estrogens and their receptors and how they are involved in the stem cell pathway.

We select a method to maintain in vitro the stem cell niches and their microenvironment, adopting appropriate conditions to limit the cell differentiation. Analyzing the mechanism of action of estrogens on thyroid cells, we observed that, at low-level, E2 favors the growth and the maintenance of the stem compartment, and in particular of CSCs. When the presence of E2 increases, cells are induced to proliferate and differentiate. In presence of hormonal imbalance, the disease can worsen going to stimulate the CSC niches, the most dangerous cellular compartment in a malignant disease. The collateral estrogen effects could manifest both in presence of local cell interaction with estrogen and with exogenous hormones, resulting in a more tumorigenic and/or metastatic malignancy. A cancer, estrogen, and stem pathways analysis showed that estrogens affect thyroid cancer both in women and in men, but with different effect. Deepening this interaction could explain the gender imbalance in tumor incidence and development for the purpose of prevent and cure thyroid pathologies with a more targeted approach.

Estrogens and Thyroid Cancer is a stem affair. A preliminary study

Introduction

Why do various pathologies show different characteristics among women and men? This could be easy to explain for diseases involving sexual organs, but how can it be justified for organs apparently similar in both genders? This is the principal question that gender medicine aims to answer.

In the thyroid gland, nodules occur with a prevalence three to four times higher in females than in males. What's more, PTC has an incidence of 3:1 when comparing women to men. In particular, this specific histotype of cancer shows an age-specific incidence in females at the beginning of the reproductive age, with a peak between 40 and 49 years. In men this peak occurs later, at 60-69 years, and is associated with a lower disease-free survival and a higher mortality rate compared to women (Rahbari, Zhang et al. 2010).

This gender discrepancy suggests a specific function of sexual hormones that could interact with thyroid function enhancing hyperproliferative and/or malignant conditions. Indeed, besides creating genetic alterations in MAPK, PI3K, and TSH signaling pathways, thyroid carcinogenesis is nurtured by the microenvironment, GFs, and hormones including estrogens (Rahbari, Zhang et al. 2010).

In the last years, different research groups have started to investigate the role of these molecules in the development of thyroid pathologies (Furlanetto, Nguyen et al. 1999; Manole, Schildknecht et al. 2001; Lee, Chen et al. 2005; Zeng, Chen et al. 2007; Ceresini, Milli et al. 2008; Kumar, Klinge et al. 2010; Rajoria, Suriano et al. 2010; Vaiman, Olevson et al. 2010; Di Vito, De Santis et al. 2011; Dong, Zhang et al. 2013). Estrogens are involved in cellular processes such as growth, cell motility, and apoptosis. They are present in both women and men with a notable increase in women in their reproductive age (Santin and Furlanetto 2011; Antico Arciuch and Di Cristofano 2012). E2 is the most potent estrogen being that it has the highest affinity to its receptors: soluble intracellular nuclear Estrogen Receptors ER-α and ER-β, and a transmembrane intracellular non-classical ER GPR30 (Santin and Furlanetto 2011; Antico Arciuch and Di Cristofano 2012). ER-α is the key factor of E2-induced proliferation with an anti-apoptosis effect. Contrarily, ER-β is associated with apoptosis and growth inhibition. In light of this, the ER-α/ER-β ratio is helpful to elucidate the TC pathophysiology (Leitman, Paruthiyil et al. 2010; Santin and Furlanetto 2011). It has been reported that circulating estrogens are directly responsible for increased susceptibility of female mice to thyroid disease, activating PI3K pathway, inhibiting p27, and affecting the transcriptional regulation of thyroid genes (i.e., TPO, DUOX1, and NIS) (Antico-Arciuch, Dima et al. 2010). Despite this and other studies have demonstrated a strong direct effect by estrogens on thyroid growth and function, the specific dynamics that move the development and in particular the initiation of proliferative and neoplastic disorders still remains to be clarified.

With regard to the trigger factors of cancer, the more plausible seems to be the

involvement of SCs in tumor formation of thyroid malignancies. In cancer initiation, the "cell-of-origin" concept explains how a normal cell acquires the first mutation, which is able to kick start the tumor, and then is defined Tumor-Initiating Cell (TIC) (Visvader 2011). Some of the best TIC candidates are SCs, able to live long and self-renew, and, most importantly, they possess the key features required for the acquisition of genetic or epigenetic changes leading to cancer development (Visvader and Lindeman 2010). Other putative TICs are committed progenitor or precursor cells, able to reacquire stem cell-like phenotype and functions upon genetic or epigenetic reprogramming (Chaffer, Brueckmann et al. 2011).

It has been reported that estrogens are involved in increasing hematopoietic SC self-renewal in female subjects and more specifically pregnant females (Nakada, Oguro et al. 2014). Xu et al. analyzed whether thyroid SC/progenitors, which express ERs, could be similarly affected by estrogen action (Xu, Chen et al. 2013). SCs that were isolated from goiter tissue enhanced their sphere-forming ability in presence of E2. Moreover, thyroidsphere cells showed ER-α mRNA levels eight times higher than those of more differentiated thyrocytes. This suggests the gender discrepancy in TC incidence, and a difference in terms of aggressiveness and survival.

Other data underline the pivotal role of estrogens in SC compartment maintenance, via the involvement of the Wnt pathway. Wnt plays a crucial role in SC/progenitor compartment maintenance, and has been described in thyroid tumors where acts through nuclear β-catenin-induced proliferation (Todaro, Iovino et al. 2010; Sastre-Perona and Santisteban 2012; Van Camp, Beckers et al. 2014). In normal and tumor thyroid cell lines, Rajoria et al. documented that E2 is associated with increased proliferation, adhesion, invasion, and migration via β-catenin (Rajoria, Suriano et al. 2010) and MMP-9 modulation (Rajoria, Suriano et al. 2011). Likewise, E-cadherin down-regulation and β-catenin translocation sustain the metastatic activity of TC cells (Dong, Zhang et al. 2013). These results confirmed the findings by Kouzmenko et al., which reported the first evidence of cross-talk between estrogens and Wnt pathways through functional interaction of β-catenin with ER-α (Kouzmenko, Takeyama et al. 2004).

This study aims to contribute to the understanding of the pathogenesis and the underlying causes of gender differences in TC. This will be obtained by studying the specific role of estrogens and their receptors and how they are involved in the stem cell pathway.

Experimental Methods and Procedures

Five-week-old nonobese diabetic/severe combined immunodeficiency mice from Charles River Laboratories were maintained in accordance to the institutional guidelines of the University of Palermo Animal Care Committee.

Sample collection and cell isolation

TC tissues were obtained at the time of total thyroidectomy from 39 patients affected by PTC (33 women and 6 men, age range 19-74 years, mean age 45 ± 14.8 years), operated at Surgical Clinic 2, University of Padua, in accordance with the ethical standards of the institutional committee on human experimentation. Diagnosis was based on the histological analysis of thyroid specimens determining size, tumor progression and the involvement of regional lymph nodes. Staging was established according to the UICC TNM classification of malignant tumor (Sobin, Gospodarowicz et al. 2009). The study protocol was reviewed and approved by the local Ethics Committee (protocol number 448 P) and each patient provided written informed consent.

Tumor tissues were washed 3-5 times in a PBS solution containing Antibiotic-Antimycotic (Euroclone®), Penicillin-Streptomycin (Euroclone®), Gentamicin (Euroclone®), and Metronidazole. An enzymatic and mechanical digestion was performed, using Collagenase II (1.5 mg/ml, Gibco®) and Hyaluronidase (20 µg/ml, Sigma Chemicals) in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich®) in agitation for 25 min at 37 °C.

Cell culture and estrogen treatments

To obtain primary cultures enriched in thyroid CSCs, cells were plated in attachment or in ultra-low adhesion in Stem Cell Medium (SCM) composed of Advanced DMEM/F-12 (Gibco®), B-27 Supplement without Vitamin A (Gibco®), N-2 Supplement (Gibco®), N-acetyl-L-cysteine (1 mM, Sigma-Aldrich®), Nicotinamide (10 mM, Sigma-Aldrich®), HEPES (10 mM, Sigma-Aldrich®), L-glutamine (2 mM, Euroclone®), Penicillin-Streptomycin (Euroclone®), supplemented with 5% Fetal Bovine Serum (Euroclone®), Recombinant Human EGF (20 ng/ml, PeproTech), and Recombinant Human FGF-basic (10 ng/ml, PeproTech). All cell culture was carried out at 37 °C in a 5% CO_2 humidified incubator.

For estrogen treatment, cells were starved overnight in DMEM/F-12, no phenol red (Gibco®) without serum, and then treated for different 17β-Estradiol (Sigma-Aldrich®) concentrations and administration time, in the same medium supplemented with 5% of charcoal stripped ultracentrifugated FBS. The different experimental tests were carried out using Recombinant Human Noggin (100ng/ml, R&D systems™) and b-FGF at different concentrations (10 and 40 ng/ml).

Flow Cytometry and ALDH activity assay

Cells were stained with conjugated Oct-3/4-PE (BD #561556 PE Mouse monoclonal anti-OCT3/4) and Sox2-FITC (BD #560301 Alexa Fluor® 488 Mouse monoclonal anti-SOX2), or with primary Estrogen Receptor α (NCL-L-ER-6F11; mouse IgG1; Novocastra™), Estrogen Receptor β1 (PPG5/10; mouse IgG2a; Dako), GPR30 (NBP1-31239; rabbit IgG; Novus Biologicals), PAX8 (ab13611; goat IgG; abcam®), TTF1 (NCL-L-TTF-1; mouse IgG1, kappa; Novocastra™) antibodies conjugated with anti-mouse or anti-rabbit Alexa Fluor® 488 (Life Technologies™). Alternatively, cell were stained with isotype-matched control. Cells were rinsed and analyzed by flow citometry using BD FACSAria™ II.

Purification of thyroid cells with a high ALDH enzymatic activity was performed using the ALDEFLUOR™ Kit (Stemcell Technologies). Cells obtained from freshly dissociated cancer thyroid tissues were re-suspended in the ALDEFLUOR assay buffer containing the ALDH substrate BODIPY®- aminoacetaldehyde (BAAA, 1 µmol/L x 10^6 cells) and incubated for 40 min at 37 °C. As negative control, an aliquot of cells of each sample was treated with 50 mmol/L of diethylaminobenzaldehyde (DEAB), a specific ALDH inhibitor. Intracellular fluorescent product was measured by flow cytometry.

Immunofluorescence and Histochemistry

For immunofluorescence assay, cells were plated on coverslips and cultured in SCMplus (SCM complemented with 100 ng/ml Noggin and 10 ng/ml FGF-2). After treatment, adhesive cells were washed with PBS, fixed with 2% paraformaldehyde at 37 °C for 20 min, permeabilized by 0.1% Triton X-100 in 0.1% sodium citrate for 15 min in ice, and then rinsed with PBS. The coverslips were exposed overnight at 4 °C to antibodies against β-catenin (H-102; rabbit polyclonal IgG; Santa Cruz Biotechnology, inc.), Estrogen Receptor α (NCL-L-ER-6F11; mouse monoclonal IgG1; Novocastra™), Estrogen Receptor β1 (PPG5/10; mouse monoclonal IgG2a; Dako), Nanog (N-17; goat polyclonal IgG; Santa Cruz Biotechnology, inc.), Oct-3/4 (C-10; mouse monoclonal IgG2b; Santa Cruz Biotechnology, inc.), PAX8 (ab13611; goat polyclonal IgG; abcam®), Sodium Iodide Symporter (SPM186; mouse monoclonal IgG1; abcam®), Sox2 (09-0024; rabbit polyclonal IgG; Stemgent®), Thyroglobulin (EPR9730; rabbit monoclonal IgG; abcam®), Thyroid Peroxidase (EPR5380; rabbit monoclonal IgG; abcam®), TSHR (4C1; mouse monoclonal IgG2a; Santa Cruz Biotechnology, inc.), TTF1 (NCL-L-TTF-1; mouse monoclonal IgG1, kappa; Novocastra™), or isotype-matched control. Unbound antibodies were removed with a washing buffer composed by PBS with 0.05% Tween-20 and 3% BSA, followed by incubation with conjugated secondary antibodies (Life Technologies™) plus RNAse (200 µg/ml, Sigma-Aldrich). Nuclei counterstaining was performed using Toto-3 iodide at room temperature for 10 min (642/660, Molecular Probes, Invitrogen). Confocal analysis was performed in order to acquire fluorescence imaging.

Histochemistry was performed on 5 µm-thick paraffin-embedded sections of thyroid specimens. For H&E staining, slides were stained in hematoxilin for 1 min, water

washed, and then exposed to eosin for 30 sec. H&E- and Alcian bleu-stained section were dehydrated and mounted in synthetic resin.

Animals and Subcutaneous Tumor Model

Subcutaneous xenografts were obtained by the injection of CSC-primary culture cells (1.5×10^6) in immunocompromised NOD/SCID Il2rg -/- female mice, alone or in combination with estrogen therapy (17β-Estradiol SE-121, 1.7 mg/pellet 60-day release for Immuno-Deficient Mice, Innovative Research of America). Tumor size was calculated each week by the appearance up to six weeks, according to the formula: $(\pi/6) \times (\text{larger diameter}) \times (\text{smaller diameter})^2$.

Migratory potential was evaluated through intrasplenic injection in NOD/SCID female mice of 3×10^5 luciferase (LUC)/GFP-transduced cells, untreated and treated with 1 nM E2 for 24 h. In order to localize and quantify dynamically the optical signal-bioluminescence in a non-invasive localization of the luciferase-marked cell population, D-luciferin (150 mg/kg, Promega) was i.p. injected at time 0 and up to 6 weeks, 5 min before the bioluminescence analysis. Image detection was performed by Biospace® instrument.

Real-Time PCR

Total RNA extraction and transcription was performed using RNeasy® Mini kit (Qiagen®) and RT^2 First Strand Kit (Qiagen®) following manufacturer's instructions. Evaluation of estrogens, cancer, and cancer stem cells target genes was performed using RT^2 profiler PCR arrays (PAHS-005Z, PAHS-033Z, and PAHS-176Z, Qiagen®) with the Rotor-Gene Q (Qiagen®). Arrays were run for E2 treated and untreated samples. Cycle threshold were normalized using ACTB, B2M, GAPDH, and RPLP0 housekeeping genes. We set a threshold of 0.02 in logarithmic scale for data analysis.

Statistical Analysis

Data were expressed as percentage ± standard deviation of the mean. Statistical significance was determined by Analysis of Variance (one-way or two-way) with Bonferroni post-test. Results were considered significant when p values were less than 0.05.

Fold-Change (2^(- Delta Delta Ct)) is the normalized gene expression (2^(- Delta Ct)) in the Test Sample divided the normalized gene expression (2^(- Delta Ct)) in the Control Sample. Fold-Regulation represents fold-change results in a biologically meaningful way. Fold-change values greater than one indicate a positive- or an up-regulation, and the fold-regulation is equal to the fold-change. Fold-change values less than one indicate a negative or down-regulation, and the fold-regulation is the negative inverse of the fold-change.

The scatter plot compares the normalized expression of every gene on the array between two groups by plotting them against one another to quickly visualize large gene expression changes. The central line indicates unchanged gene expression. The boundary (fold regulation cut-off) was set at 2.

Results

Thyroid tumor bulk contains stem-like cells

In recent years, remarkable achievements in thyroid carcinogenesis have led to the isolation and characterization of CSCs. In both differentiated and undifferentiated histotypes, tumorigenic potential is confined in a subpopulation of stem-like cells expressing high Aldehyde Dehydrogenase ($ALDH^{high}$) activity. The ALDH activity is important for several biological functions, participating in early cellular differentiation, detoxification, and drug resistance, through the oxidation of intracellular aldehydes (Moreb, Ucar et al. 2012).

ALDH activity is highly expressed in stem and progenitor cells, thus it has been used as functional marker for CSCs isolation in tumors (i.e., breast, lung, ovarian, prostate, head-neck, and thyroid cancer), as well as in multiple myelomas and acute leukemia (Klonisch, Hoang-Vu et al. 2009; Marcato, Dean et al. 2011). In a study published in 2010, our group observed a progressive increase of $ALDH^{high}$ subpopulation in the more aggressive tumor histotypes (FTC: 3 ± 1%; PTC: 7 ± 2%; ATC: 16 ± 4%) (Todaro, Iovino et al. 2010). $ALDH^{high}$ cells could be expanded indefinitely in vitro, because of its unlimited replication potential. When transplanted in immunodeficient mice, these $ALDH^{high}$ cells retain the tumor-initiating capacity and reproduce the phenotypic characteristics of the parental tumor. In particular, orthotopic injections of 5,000 $ALDH^{high}$ cells in nude mice were more tumorigenic than 25,000 $ALDH^{low}$ cells or than 25,000 unsorted cells from tumor bulk. Moreover, the injection of $ALDH^{high}$ cells derived from FTC led to a moderately invasive tumor without distant metastasis, while $ALDH^{high}$ cells derived from ATC developed invasion of local tissues (i.e., esophagus, trachea) and lymph nodal and lung metastasis, correlated with a constitutive activation of cMET/AKT pathways (Todaro, Iovino et al. 2010).

Elevate ALDH activity was identified also in ATC cell lines: 17-38% of ATC-8505C (Klonisch, Hoang-Vu et al. 2009) and 8-13% of SW1736 (Carina, Zito et al. 2013) were $ALDH^{high}$. We tested the ALDH activity in two different thyroid cell lines, K1 and BCPAP, founding a different amount of cells harboring the relative fluorescence. In K1 the percentage of $ALDH^{high}$ cells was 23,6 %, while in BCPAP it is 4,87% (**Figure 1a**).

Nowadays, cell surface markers specific for thyroid CSCs have not yet been identified. Rather than performing a cell sorting, we preferred to work with primary cultures highly enriched in CSCs isolated from PTC patients. Thyroid CSCs are capable of growing as sphere-like aggregates in a serum-free culture medium, enriched with hormones and supplemented with EGF and FGF-2; indeed, if placed in ultra-low attachment plates, thyrosphere can survive because of their anchorage-independent growth. This way, it is easier to isolate CSCs from differentiated thyrocytes and fibroblasts inhibited by the absence of serum and by non-adherent conditions.

In order to find the best method of thyroid CSC isolation, we tested the survival of these cells in the presence or absence of serum, using different laboratory plastics (**Figure 1c**). Both in ultra-low adhesion and attachment conditions, thyroid cells tend to form

spheres in culture. What we notice is that, in the long run, the suspension cells could survive better in the presence of adherent cells. Probably, the other cells constitute a kind of microenvironment that allows the release of certain factors necessary for their growth. In ultra-low conditions in presence of 10% of serum, the cells tend to agglomerate to take the form of a primordial follicle, containing a colloidal-substance. To find the right balance between the in vitro niches maintenance and the undifferentiated state preservation, we did several tests at different serum concentration. In conclusion, the optimization of the method led us to choose a 5% serum medium for the culture conditions in attachment.

We then verified the presence of CSCs in the isolated culture through the analysis of stemness biomarkers. Nanog, Oct-4, and transcription factor SOX-2 (encoded by NANOG, POU5F1, and SOX2 genes, respectively) are transcription factors that work together through a pluripotent regulatory network to establish SC features, maintaining pluripotency and self-renewal capability (Rodda, Chew et al. 2005). Analysis of over-expression of these markers can help to identify putative thyroid CSCs (Friedman, Lu et al. 2009; Klonisch, Hoang-Vu et al. 2009; Todaro, Iovino et al. 2010; Zheng, Cui et al. 2010; Tseng, Huang et al. 2012; Li, Reeb et al. 2013).Thyroid CSCs identification can be supported by the analysis of biomarkers belonging to self-renewal control pathways, such as Wnt/β-catenin, Hedgehog and Notch (classified also as EMT-inducing signaling pathways)(Mitsutake, Iwao et al. 2007). Then, in the first analysis, we investigate the expression of Sox2, Oct3/4, and β-catenin on thyroid cells isolated from fresh tumor tissue. We observed a clear expression of these markers, in particular Oct3/4, in thyroid tumor cells. The thyroid tissue derivation was confirmed by the presence of TSH-R on these cells (**Figure 1b**).

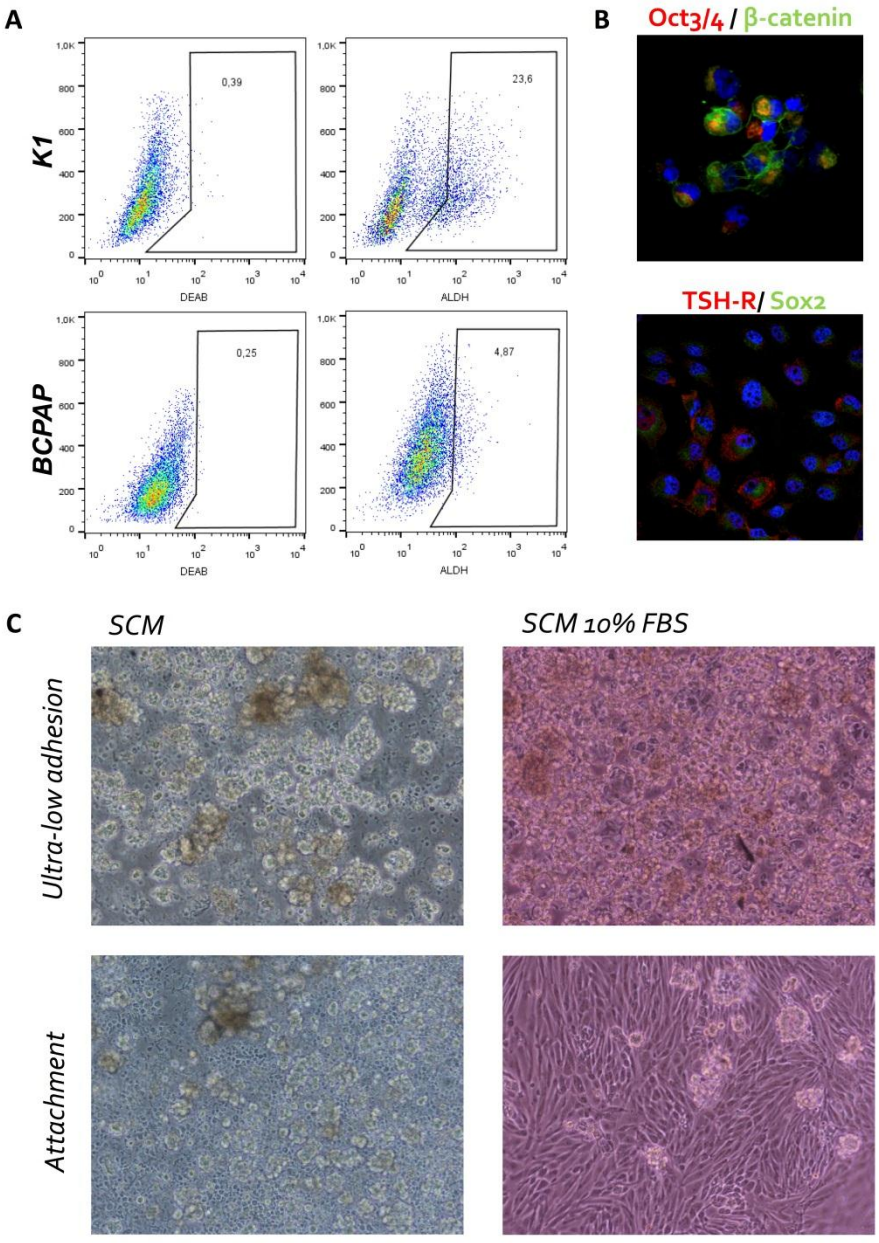


Figure 1. Thyroid tumor bulk contains stem-like cells

(A) Percentage of ALD^{high} cells within K1 and BCPAP cell lines.

(B) Immunofluorescence analysis of Sox2, Oct3/4, β-catenin, and TSH-R on primary cell cultures.

(C) Optimization of isolation methods. Cell culture maintained in attachment and ultra-low adhesion, in the presence or absence of FBS.

BMP pathway inhibition acts with FGF-2 in maintenance of stemness in vitro

The previous described method was used to isolate CSC-enriched cultures from PTC patients, after enzymatic and mechanical digestion. We carried on the experiments on tissues derived from male and female PTC patients, at early and late stage (**Figure 2a**). Among them, we selected primary cell lines enriched in thyrospheres, able to maintain the double population -adherent and suspension- in culture (**Figure 2b**).

Given the difficulty ok keeping the undifferentiated state for a long time in culture, we adopted conditions limiting the cell maturation, drawing inspiration from media and complements used for embryonic SCs. Some authors tested various complements in order to find the best combination of factors able to maintain the undifferentiated growth of human embryonic SCs in the absence of feeder layers (Wang, Zhang et al. 2005; Liu, Song et al. 2006). Wang et al. found that BMP antagonist Noggin and FGF-2 was sufficient to keep a prolonged growth of embryonic SCs retaining their features. This is consistent with the study by Longmire et al., which have deepened the different steps of follicular cell development, from embryonic SCs to thyroid progenitors (Longmire, Ikonomou et al. 2012). They demonstrated that the presence of Activin A led embryonic SCs to differentiate in Definitive Endoderm. At this stage, if exposed to BMP and Activin/TGFβ signaling inhibitors (Noggin and SB431542, respectively) for a specific amount of time, definitive endoderm were directed towards the Lung/Thyroid Competent Definitive Endoderm. Hence, induction of BMP and FGF signaling by exposing cells to high doses of FGF-2, combined with specific factors, promoted initial lineage specification of endodermal TTF-1+ Thyroid Primordial Progenitors.

Based on these studies, we tested the role of BMP inhibitor and FGF-2 on cultured cells, looking for the right balance between undifferentiated state, growth, and thyroid derivation. We treated tumor cells with 10 ng/ml FGF-2, 100 ng/ml Noggin and 10 ng/ml FGF-2, or 100 ng/ml Noggin and 40 ng/ml FGF-2. After 48 h, the cells were analyzed at FACS instrument for SC (Sox2 and Oct3/4) and progenitors (Pax8 and Ttf-1) markers. We also investigated the modulation of ERs expression, in particular of ER-α and ER-β. We found that the presence of Noggin and FGF-2 in culture enhances the stem/progenitor cells markers expression, in particular at concentration of 100 ng/ml for Noggin and 10 ng/ml for FGF-2. These conditions also lead to an increase of both ERs, leading to a ER-α/ER-β ratio towards growth and anti-apoptosis function. We then developed a culture medium called Stem Cell Medium plus (SCMp), composed of SCM with the addition of 100 ng/ml Noggin and 10 ng/ml FGF-2, which was used for all subsequent experiments.

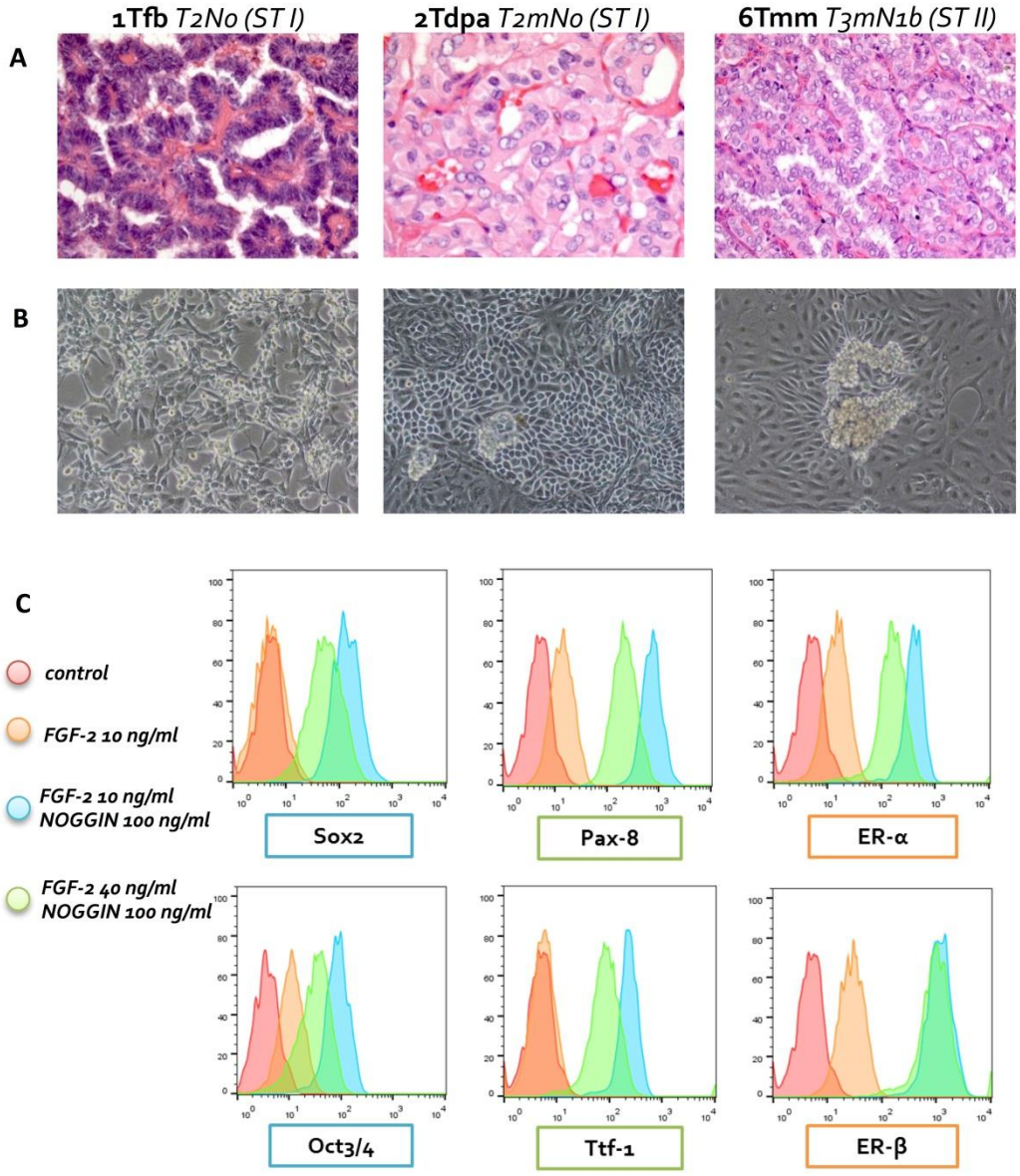


Figure 2. BMP pathway inhibition acts with FGF-2 in maintenance of stemness in vitro

(A) H&E staining of paraffined PTC samples (magnitude 40x, 40x, 63x, respectively).

(B) CSC-enriched cell cultures isolation.

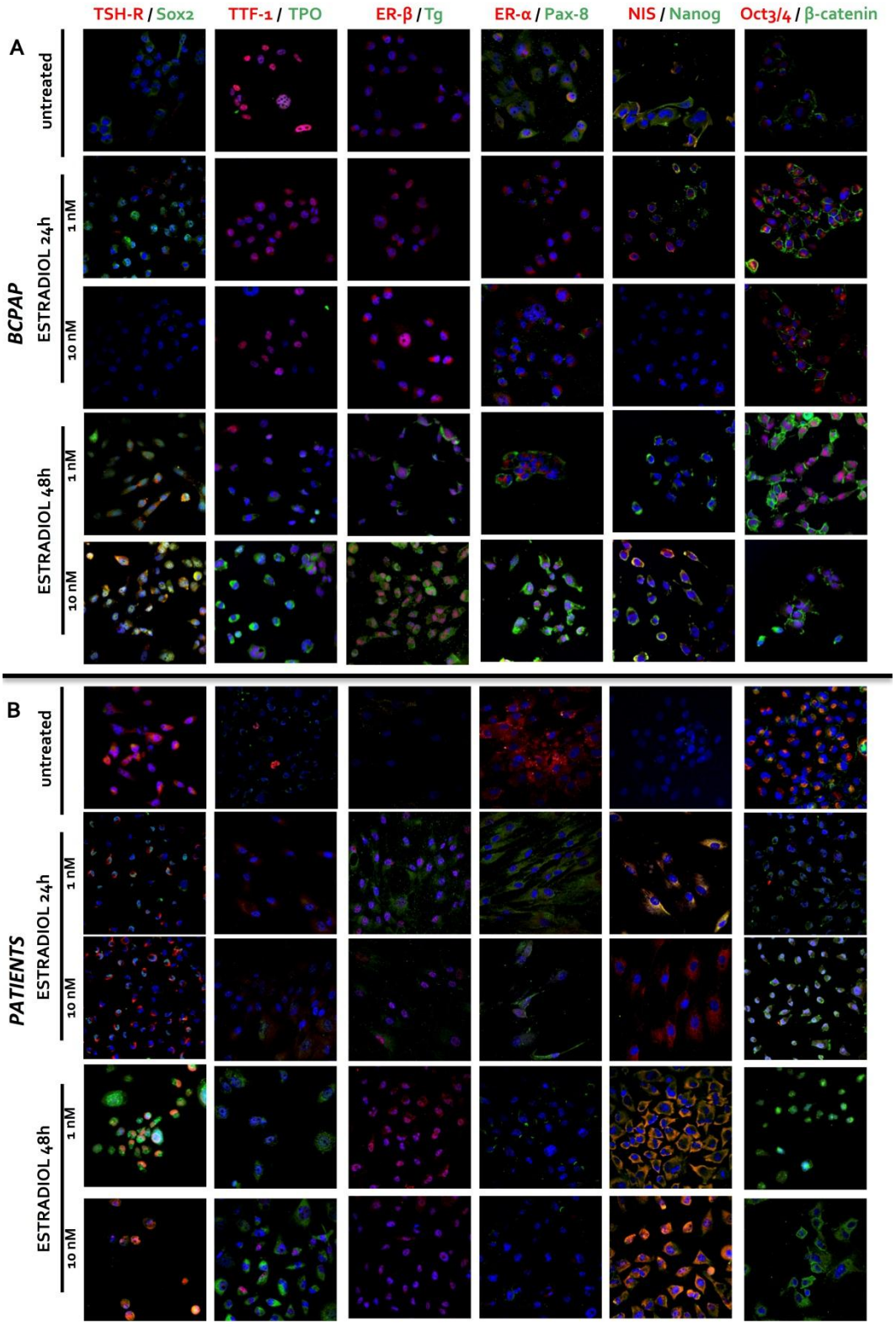
(C) FACS analysis on CSC-enriched cell cultures for Sox2, Oct3/4, Pax8, Ttf-1, ER-α and ER-β after treatment with FGF-2 and Noggin.

Estrogen treatment enhances stem markers expression in a time- and dose-dependent manner

Since ERs were identified in benign and malignant thyroid tissues, different research groups approached to investigate the role of estrogens in TC proliferation and migration (Manole, Schildknecht et al. 2001; Lee, Chen et al. 2005; Vivacqua, Bonofiglio et al. 2006; Zeng, Chen et al. 2007; Rajoria, Suriano et al. 2010; Xu, Chen et al. 2013). Each of them, however, has used different E2 concentrations and administration time. Then, we performed a immunofluorescence panel in order to analyze the presence and the localization of SC (Oct3/4, Sox2, Nanog, β-catenin), progenitors (Pax8, Ttf-1), differentiation (TSH-R, TPO, Tg), and estrogen (ER-α, ER-β) markers. This panel has been designed to analyze CSC-enriched primary cultures and BCPAP cell line as control after 1 nM and 10 nM E2 treatment for 24h and 48h repeated administration (**Figure 3a-b**).

About stem markers, Sox2, Oct3/4, and Nanog enhance their expression after treatment, localizing at nuclear or perinuclear level, in particular with 1 nM E2 administration. At 48h treatment, Oct3/4 expression is not present in patient cells (**Figure 3b**). Similarly, β-catenin changes their localization, being nuclear with 1 nM E2 and citoplasmatic at 10 nM E2. Comparing BCPAP and CSC-enriched patients culture cells, in differentiated tumor cells β-catenin appears more limited to the membrane. Progenitor marker Ttf-1 increases its expression with 10 nM E2, in particular after 48h, in patient cells (**Figure 3b**). Also Pax8 expression enhance after 48h, as we can observe in particular in BCPAP (**Figure 3a**). Differentiation markers (TSH-R, TPO, and Tg) increase their expression after 10 nM treatment and in particular after 48h. Finally, ER-α appears more evident in untreated and after 1 nM 24h E2 treatment. Contrarily, ER-β passes from perinuclear in untreated cells and after 1nM 24h E2 treatment to a more nuclear localization after 48h E2 treatment.

Summing up the results, we observed that 1nM 24h E2 treatment guarantees a marked expression of SC markers, while 10 nM E2 48h E2 treatment promotes an increase of expression of progenitors and differentiation markers, as we can confirm with the FACS analysis (**Figure 3c**). Moreover, ER-α expression and then its pro-proliferation and anti-apoptotic function are increased in untreated and 1 nM 24h treated cells. Contrariwise, ER-β enhances its expression with 10 nM E2 treatment, and more important passes from perinuclear at nuclear localization. This implies a more active form of ER-β and then a major inclination for anti-proliferation and pro-apoptosis effect. In conclusion, ERs seem to be dose- and time-dependent, modifying their expression and localization depending on the treatment conditions. Estrogens appear dose- and time-dependent also in modulating undifferentiated state, suggesting that a continuous and consistent dose of estrogens allows the development of a protective condition against tumor.


TSH-R / Sox2
TTF-1 / TPO
ER-β / Tg
ER-α / Pax-8
NIS / Nanog
Oct3/4 / β-catenin
A
BCPAP
untreated
ESTRADIOL 24h
1 nM
10 nM
ESTRADIOL 48h
1 nM
10 nM
B
PATIENTS
untreated
ESTRADIOL 24h
1 nM
10 nM
ESTRADIOL 48h
1 nM
10 nM

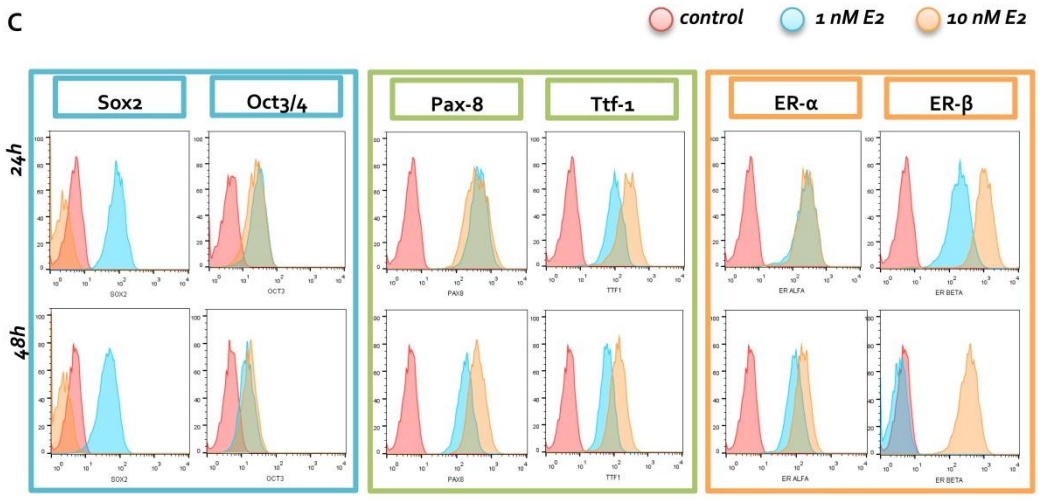


Figure 3. Estrogen treatment enhances stem markers expression in a time- and dose-dependent manner

(A) Immunofluorescence analysis on BCPAP for Sox2, Oct3/4, Nanog, Pax8, Ttf-1, TSH-R, Tg, TPO, NIS, β-catenin, ER-α and ER-β after E2 treatment at 1 and 10 nM for 24h and 48h.

(B) Same immunofluorescence analysis on CSC-enriched cell cultures.

(C) FACS analysis on CSC-enriched cell cultures for Sox2, Oct3/4, Pax8, Ttf-1, ER-α and ER-β after E2 treatment.

A continuous presence of estradiol has an opposite effect compared to a hormonal imbalance simulation

We have deepened the effect of the continuous presence of estrogens prolonging the 1 nM and 10 nM E2 treatment for 7 days, and observing the modulation of different markers after further 7 days after discontinuing the treatment. Moreover, given that clinical and epidemiological studies reported an unbalanced estrogen metabolism in TC (Rahbari, Zhang et al. 2010; Zahid, Goldner et al. 2013), we simulated an in vitro condition of hormonal imbalance.

After 7 days E2 treatment, the morphology of 1 nM and 10 nM remained essentially the same. In unbalanced conditions, instead, many cells detached from the flask and survived in suspension (**Figure 4a**).

Comparing the Mean Fluorescence Index (MFI) of Sox2, Oct3/4, and Nanog, we noted that continuous and unbalanced treatment led to an opposite behavior: continuous treatment repressed Sox2 and Oct3/4 expression, while unbalanced treatment enhanced these stem marker expression (**Figure 4b**). After discontinuing the treatment for a week, Sox2 expression decreased, while Oct3/4 notably increased in all kinds of treatment. Nanog expression was weekly modulated, but nevertheless increase after E2 treatment.

Observing ERs expression, E2 induce a decrease of ER-α in both continuous and unbalanced treatments, although with a very slight difference. Its expression changed after a week of treatment discontinuation, increasing in particular after 10 nM E2 treatment. For ER-β and GPR30, it is possible to observe the same trend, with a decrease in expression with hormonal imbalance and increase in continuous presence of E2. 7 days after discontinuing the treatment, ER-β and GPR30 expression continues to decline, leading to a reversal in the effect on thyroid tumor cells. The ER-α/ER-β ratio changed, giving a more protective effect when receiving continuous E2 administration, but was more prone to proliferation in imbalanced conditions, playing out an anti-apoptosis function.

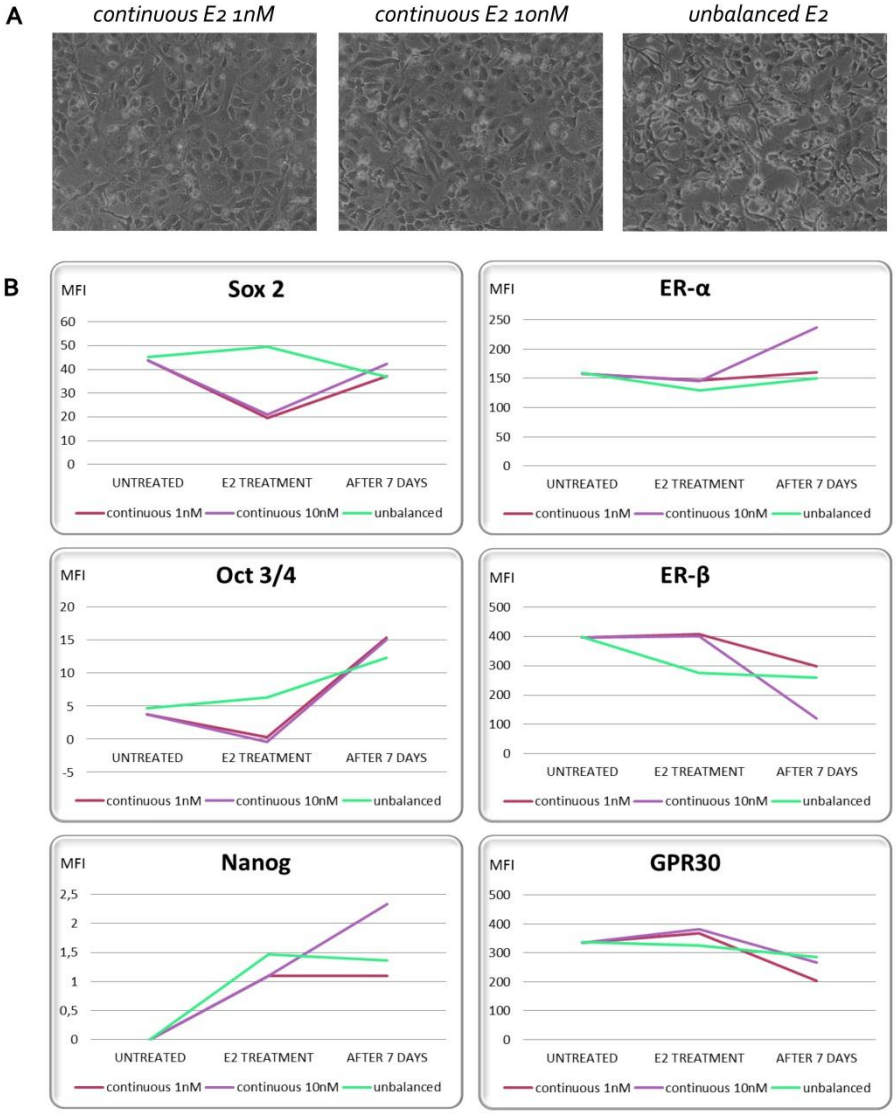


Figure 4. A continuous presence of estradiol has an opposite effect compared to a hormonal imbalance simulation

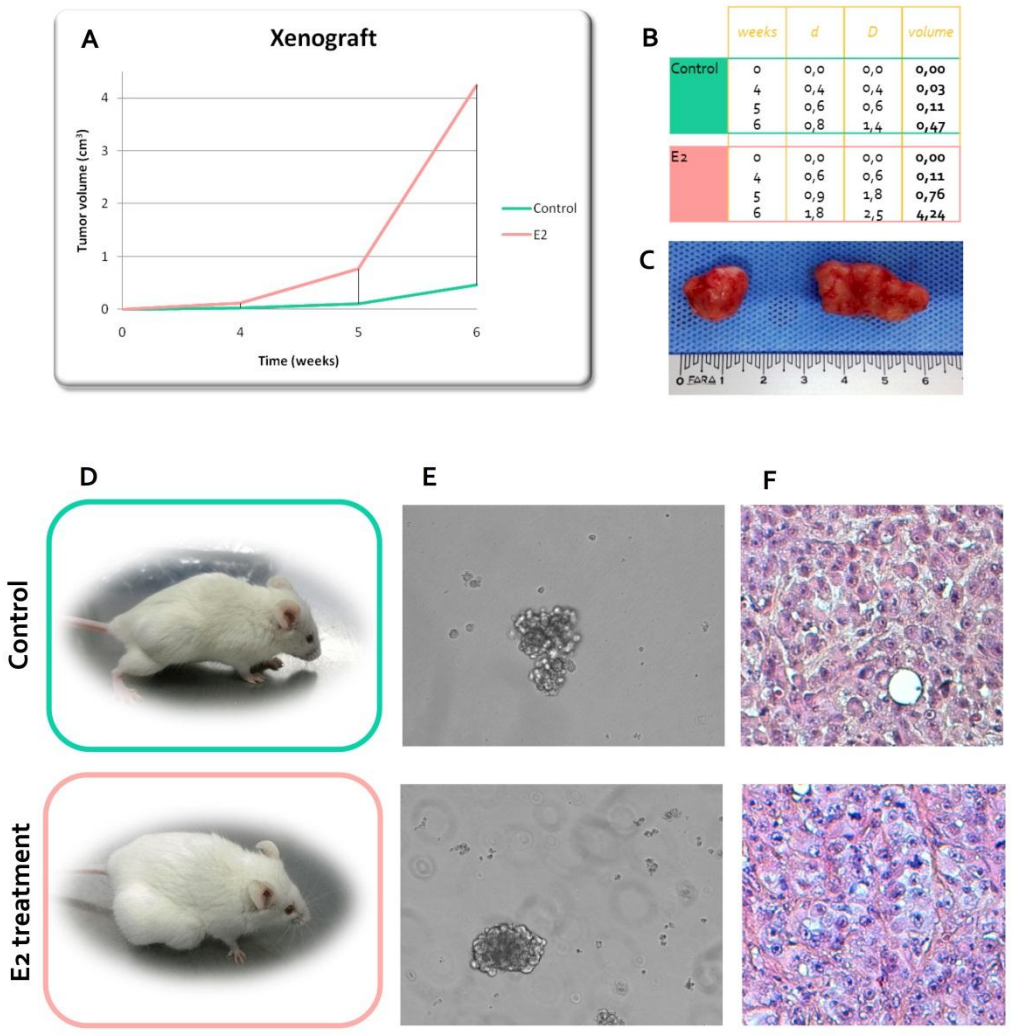
(A) Morphological evaluation of CSC-enriched cell cultures after 1 and 10 nM continuous E2 treatment and with hormonal imbalance simulation (magnitude 10x)

(B) Mean Fluoresce Index of FACS analysis for Sox2, Oct3/4, Nanog, ER-α, ER-β and GPR30.

Host estrogen therapy enhances the tumorigenic potential of Thyroid CSCs

One peculiar characteristic of CSCs is the ability to form a tumor when transplanted into immunodeficient mice. Injection of putative CSCs in immunocompromised mouse models and following the tumor development constitutes a univocal way of testing their effective tumorigenicity (Ailles and Weissman 2007). Indeed, xeno-transplantation permits to observe the ability of CSC-enriched spheroids to initiate tumor in murine models, such as nude, SCID, or NOD-SCID-IL2Rγ$^{-/-}$ (NSG) mice (Todaro, Iovino et al. 2010; Li, Reeb et al. 2013; Ahn, Henderson et al. 2014). It has been observed that serial transplantation led to an in vivo selection of cells able to generate more aggressive tumor (Clarke, Dick et al. 2006). In particular, transplantations of cells isolated from secondary and tertiary xenografts permit to define their long-term tumorigenic potential, as well as self-renewing ability.

We tested the tumorigenic capacity through subcutaneous inoculation of 1.5 x 10^6 CSC-enriched cells in immunocompromised NOD/SCID Il2rg -/- mice, alone or in combination with estrogen therapy. In both cases, injected cells gave rise to a tumor mass, confirming the presence of tumorigenic cells. After 6 weeks, thyroid tumor cells injected into mice receiving estrogen therapy developed a tumor 9 fold higher than those developed in untreated mice (**Figure 5a-d**). After digestion, xenograft-derived cells were propagated in spheroid cultures, validating their CSC-like features (**Figure 5e**). Histological examination suggested that the E2 therapy received by the hosts has led to a more differentiated tumor compared to the tumor mass grown in untreated mice (**Figure 5f**).



	weeks	d	D	volume
Control	0	0,0	0,0	0,00
	4	0,4	0,4	0,03
	5	0,6	0,6	0,11
	6	0,8	1,4	0,47
E2	0	0,0	0,0	0,00
	4	0,6	0,6	0,11
	5	0,9	1,8	0,76
	6	1,8	2,5	4,24

Figure 5. Host estrogen therapy enhances the tumorigenic potential of TC stem cells

(A) Diagram of tumor volume growth with or without host E2 therapy.
(B) Data on xenograft tumor dimensions.
(C) Macroscopic analysis of xenograft tumors.
(D) Subcutaneous xenografts obtained by the injection of CSC-primary culture cells (1.5 x 10^6) in immunocompromised NOD/SCID Il2rg -/- female mice, alone or in combination with estrogen therapy.
(E) Thyroidsphere in culture after xenograft tumor digestion.
(F) H&E staining of paraffined xenograft tumors.

E2-treated CSCs empower their migratory activity in vivo

We then analyzed the migratory capacity of CSC-enriched cells using an in vivo model. This assay was performed through intrasplenic injection in NOD/SCID mice of 3×10^5 luciferase (LUC)/GFP-transduced cells, untreated and pre-treated in vitro with 1 nM E2 for 24 h.

This in vivo study highlighted a substantial migratory capacity of the inoculated E2 treated cells. 30 min after injection, spleen was removed and observed at Biospace instrument: the untreated cells were retained for the most part in the spleen, while pretreated cells were almost entirely migrated.

After 5 weeks, E2 pretreated cells migrated to distant organs, such as lung, brain, and liver. Also untreated cells migrated, but in organs circumscribed to the abdomen and peritoneum (**Figure 6a-b**).

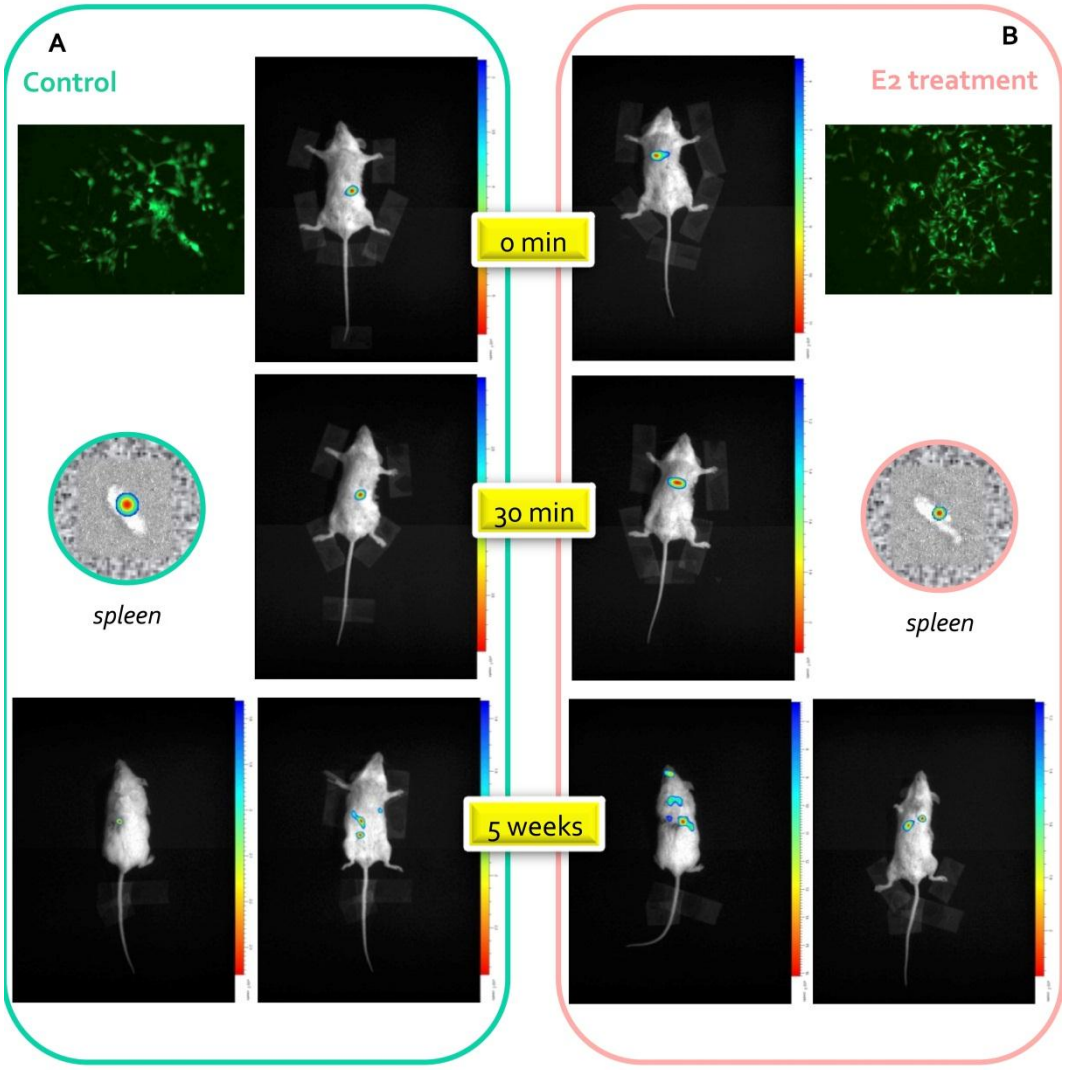


Figure 6. E2-treated CSCs empower their migratory activity in vivo

(A) Intrasplenic injection in NOD/SCID female mice of 3 x 10^5 luciferase (LUC)/GFP-transduced cells. Analysis of migratory capacity at time 0,30 min, 5 weeks.

(B) Intrasplenic injection in NOD/SCID female mice of 3 x 10^5 luciferase (LUC)/GFP-transduced cells, treated with 1 nM E2 for 24 h. Analysis of migratory capacity at time 0,30 min, 5 weeks.

Estrogens perform their function in a different way in women and men

In order to investigate the mechanism of action of estrogen, we performed a pathway analysis for factors involved in estrogen and cancer signaling, with a focus on the SC compartment. Wishing to look for an answer to the gender disparity in incidence and aggressiveness of TC, we analyzed separately female and male samples. Here, we reported a first roundup of estrogen target explored in CSCs-enriched cell lines (**Figure 7**).

First of all, we noted that in male the most expressed receptor is ERα, followed by GPR30 and ERβ. In female cells, instead, the most expressed estrogen receptor is GPR30, followed by ERβ and ERα. This indicates a preferential way chosen by estrogens to perform their mechanism of action, that seems more protective in females at basal level. After E2 treatment, male showed a down-regulation of both receptors, while female reported an over-expression of ERα. Most important, what changes is the ratio ERα/ERβ, leading to a propensity towards proliferation and anti-apoptosis stronger in females than in males.

Estrogen interacts also with GFs pathway, as evidenced by **EGF**, **ERBB3**, and **FGFR2** over-expression in patient samples, and with proliferation through Lin-28 homolog A (**LIN28A**) and B (**LIN28B**).

Treated tumors have increased CSC markers. In particular, they showed an up-regulation of ATP-binding cassette sub-family B member 5 (**ABCB5**), ATP-binding cassette sub-family G member 2 (**ABCG2**), Aldehyde dehydrogenase 1 family, member A1 (**ALDH1A1**), GATA binding protein 3 (**GATA3**), and Integrin alpha 6 (**ITGA6**), and an down-regulation of adhesion molecules, such as Platelet/endothelial cell adhesion molecule (**PECAM1,** or **CD31**). The up-regulation of DNA (cytosine-5-) methyltransferase 1 (**DNMT1**) and the absence of BMP family members indicate the propensity of cells for self-renewal. The pluripotence is maintained by the increase of **POU5F1**, **SOX2**, and **NANOG** expression, evidenced principally in females, in both cancer stem and differentiated cells. In these patients, we observed an hyperactivation of Hippo (with **LATS1** and **TAZ**), Hedgehog (**PTCH1** and **SMO**), and Notch signaling (**DLL1**, **JAG1**, **MAML1**, and **NOTCH1**). In male patients, instead, we observed a down-regulation of Hedgehog members, suggesting a different mechanism of action of hormones in this gender.

Deepening the hallmarks of cancer, we noted that the angiogenic behavior is different among patients. In particular, we observed an increase in angiogenic factors in males and an opposite decrease in females of Vascular Endothelial Growth Factor A (**VEGFA**) and Vascular Endothelial Growth Factor Receptor 1 and 2 (**VEGFR1** and **VEGFR2**). Moreover, only in females there is a down-regulation of Interleukin 8 (**IL8**) and an up-regulation of Pigment epithelium-derived factor (**SERPINF1**), a potent angiogenesis inhibitor, and of Angiopoietin 1 and 2 (**ANGPT1** and **ANGPT2**), which could have a role in blocking angiogenesis.

Also the cell cycle is differently regulated in women and in men. While in male patient we observed a down-regulation of Cyclin D2 (**CCND2**) and an up-regulation of Wee1-like protein kinase (**WEE1**), female patient showed an opposite attitude, with enhancement of Aurora kinase A (**AURKA**), Cyclin D3 (**CCND3**), Cell division cycle protein 20 homolog (**CDC20**), Ki-67 (**MKI67**), S-phase kinase-associated protein 2 (**SKP2**), Stathmin 1 (**STMN1**), and decrease of **WEE1**. This means that, if the entry in S phase is inhibited and the G2 - M progression is negatively regulated in males, the E2 treatment led to a promotion of every cell cycle phase in females.

In female, otherwise, it is evidenced an alteration in DNA repair activity, with down-regulation of DNA-damage-inducible transcript 3 (**DDIT3**), Growth arrest and DNA-damage-inducible, alpha (**GADD45**), and Protein phosphatase 1, regulatory subunit 15A (**PPP1R15A**). Similarly, the telomerase activity is enhanced by Dyskerin (**DKC1**) and Telomerase-associated protein 1 (**TEP1**) up-regulation in female.

Also about Migration and Metastasis, and then Epithelial-to-Mesenchymal Transition, we noted a modulation: DNA-binding protein inhibitor (**ID-1**), Occluding (**OCLN**), Snail homolog 1 (**SNAI1**), Twist homolog 1 (**TWIST1**) and 2 (**TWIST2**), and Zinc finger E-box binding homeobox 1 (**ZEB1**) are regulated in both genders, but with opposite behavior. In fact, it seems that EMT is favorite in male after E2 treatment, but not in female. In particular, **KLF17**, a negative regulator of EMT through the binding to ID-1, is down-regulated in male and up-regulated in female. Similarly, we observed a reduction of SNAI1, TWIST1, and TWIST2 expression in females, but not in male, where we found a decrease of Dachsund homolog 1 (**DACH1**), a TGF-β signaling inhibitor. On the other hand, the variety of markers and their modulation require further validation.

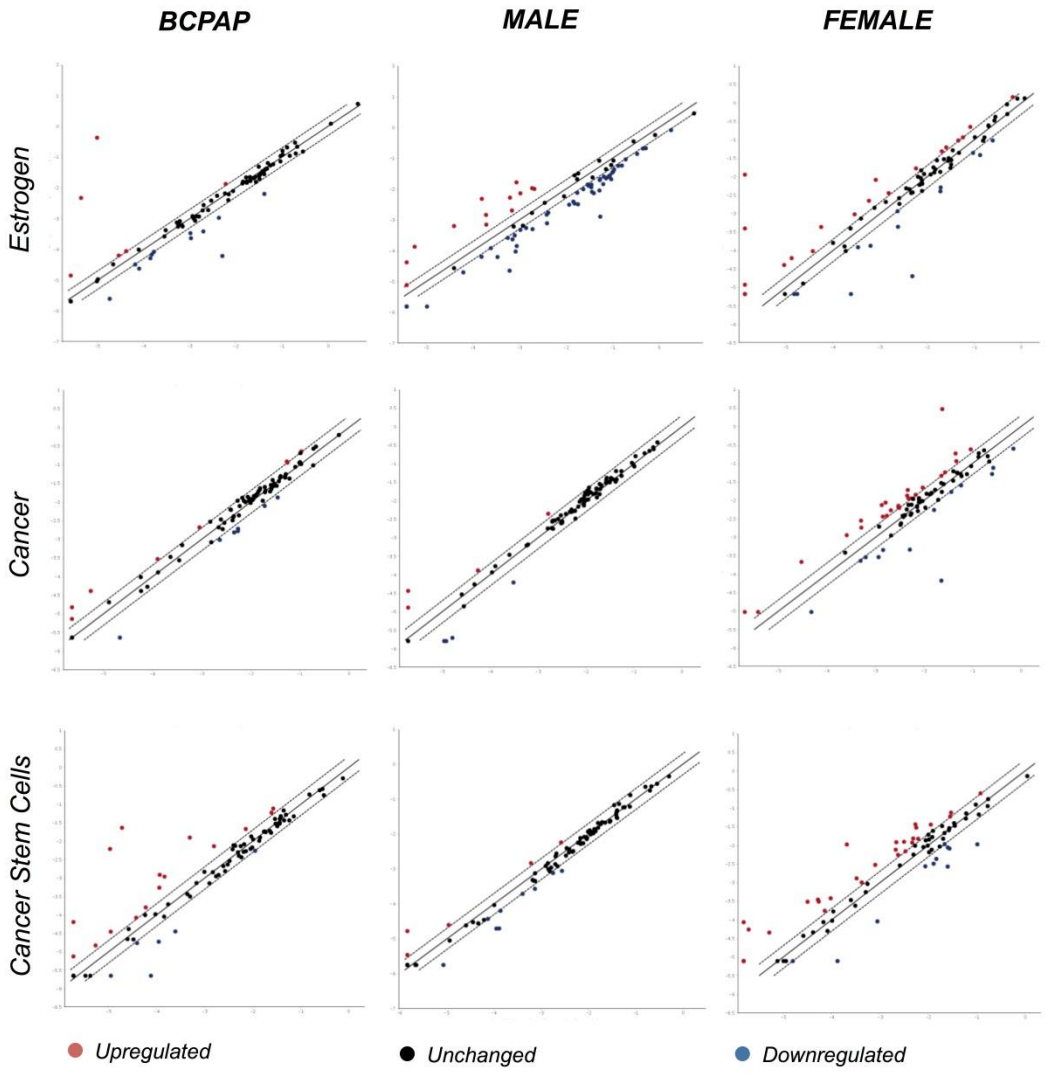


Figure 7. Estrogens perform their function in a different way among women and men
Estrogen, cancer, and cancer stem cell pathway analysis for BCPAP and female and male CSC-enriched cell cultures.

Discussion

Thyroid diseases are widespread among the population. However, they are often undervalued by the scientific point of view, since they rarely end with fatal outcome. The incidence of these pathologies affects mainly the female gender, with a frequency of 3 to 5 times higher depending on the severity degree of disease, which may not involves severe physical discomforts, but has a strong impact on quality of life.

The origin of the disorders is the loss of the balance between the many factors that regulate the functioning of our body. In particular, it has been observed that excessive or unregulated exposure to various hormones, and in particular to estrogen, may affect the course of thyroid diseases. Recent studies also demonstrate that sexual hormones could exert a supportive role in the propagation of SCs and progenitors, as suggested by the cross-talk between estrogen signaling and Wnt pathway.

In the present work, we aim to investigate the fine mechanisms regulating the interconnection between estrogens, thyroid, and SCs. We identified the presence of CSCs in primary cultures isolated from PTC patients, characterized by elevate ALDH activity and by the expression of stem markers (Figure 1). We proved that the presence of GFs, such as FGF-2, and the inhibition of BMP pathway interact with the growth of CSCs, preventing their differentiation (Figure 2).

Deepening the mechanism of action of estrogens on thyroid cells, we observed that, at low-level E2 (i.e., 1 nM), E2 favors the growth and the maintenance of the stem compartment, and in particular of CSCs. When the presence of E2 increases until 10 nM, cells are induced to proliferate and differentiate (Figure 3). Then, if we are in the presence of hormonal imbalance, the disease can worsen going to stimulate the CSC niches, the most dangerous cellular compartment in a malignant disease (Figure 4).

The collateral estrogen effects could manifest both in presence of local cell interaction with estrogen and when we administer exogenous hormones, resulting in a more tumorigenic and/or metastatic malignancy (Figure 5 and 6).

What is surprising is that estrogens affect TC both in women and in men, but with different – and in some cases opposite – effect. Estrogens modulate differently their mechanism of action through the choice of receptors with which they interact. They perform a different modulation of stem compartment, enhance the activity of transcription factors and stem-related pathways in women, but limiting them in men. Also cancer function are altered in a gender-specific way, like angiogenesis, cell cycle, DNA-repair activity and EMT (Figure 7).

This is a preliminary study that aims to take a look on the concert of factors intervening in thyroid malignancies, and in particular to focus on the causes that underlie of gender discrepancy. Deepening this interaction could explain the gender imbalance in tumor incidence and development for the purpose of prevent and cure thyroid pathologies with a more targeted approach.

Estrogens and Thyroid Cancer is a stem affair. A preliminary study

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